CIB1 MEDIATED REGULATION OF ENDOTHELIAL CELLS AND PATHOLOGICAL ANGIOGENESIS

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ABSTRACT

Mohamed A. Zayed: CIB1 Mediated Regulation of Endothelial Cells and Pathological Angiogenesis

(Under the direction of Leslie V. Parise, Ph.D.)

Calcium and integrin binding protein 1 (CIB1), a 22kDa EF-hand containing protein, that was originally identified to bind the platelet integrin αIIb, can also bind and regulate various other proteins. Among the newly identified CIB1-binding partners is the p21-activated kinase 1 (PAK1), which is known to regulate ECs and contribute to angiogenesis *in vivo*. Since CIB1 is present in highly vascularized tissue and is expressed in various types of ECs, we hypothesized that it may also have an important role in vascular tissue. The work described herein is a collection of studies that for the first time investigates the role of CIB1 in EC signaling, function, and angiogenesis.

Pathological angiogenesis contributes to various ocular, malignant, and inflammatory disorders, emphasizing the need to understand this process on a molecular level. We demonstrate here that CIB1 is necessary for various EC functions such as migration, proliferation, tubule formation, and monolayer permeability. CIB1 also regulates PAK1 activation, as well as downstream ERK1/2 phosphorylation and MMP-2 expression. Depletion of CIB1 in ECs attenuates their response to angiogenic growth factors such as VEGF and bFGF. In *ex vivo* and *in vivo* assays, CIB1-KO tissue also has an attenuated

response to growth factors, demonstrating that CIB1 is necessary for a robust angiogenic response. Moreover, although we confirm that CIB1-KO mice have no defects in developmental vasculogenesis and angiogenesis, we demonstrate that CIB1 is essential for ischemia-induced and tumor-induced pathological angiogenesis. These findings are important since they differentiate between physiological and pathological forms of angiogenesis and identify CIB1 as novel target for pro- and anti-angiogenic therapy.

DEDICATION

To my parents, Nadia and Adel, for their guidance, support, encouragement, and who instilled in me my love for learning, my passion for science, and my drive to excel. And, to my wife, Nuha, for her endless love and everlasting support.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
aFGF	Acidic fibroblast growth factor
AMD	Age-related macular degeneration
BAEC	Bovine aortic endothelial cell
bFGF	Basic fibroblast growth factor
CIB1	Calcium and integrin binding protein 1
DIC	Differential interference contrast
EC	Endothelial cell
ECM	Extracellular matrix
ED	Embryonic day
EDCC	Endothelial-derived circulating cell
EDG1	Endothelial differentiation sphingolipid G-protein-coupled-receptor-1
EGF	Epidermal growth factor
EHS	Engelbreth-Holm-Swarm
eNOS	Endothelial nitric oxide synthase
FAK	Focal adhesion kinase
FGFR	FGF receptor
GCL	Ganglion cell layer
GFR	Growth factor reduced
HAEC	Human aortic endothelial cell

HCAEC	Human coronary artery endothelial cell
hCG	human chorionic gonadotropin
HGF	Hepatocyte growth factor
HHT	Hereditary hemorrhagic telengiectasia
HIF-1	Hypoxia inducible factor-1
HIV-1	Human immunodeficiency virus type-1
HPAEC	Human pulmonary artery endothelial cell
HSC	Hematopoetic stem cell
HSPG	Heparin sulfate proteoglycans
HUVEC	Human umbilical vein endothelial cell
ICAM	Intracellular adhesion molecule
ILM	Inner limiting membrane
$InsP_3R$	Inositol 1,4,5-triphosphate receptor
IPL	Inner plexiform layer
ITC	isothermal titration calorimetry
KO	Knockout
MAPK	MAP kinase
MCP1	Monocyte chemoattractant protein-1
MEC	Mouse intraembryonic endothelial cell
MHEC	Mouse heart endothelial cell
MLEC	Mouse lung endothelial cell
MMP	Matrix metalloproteinase
MT-MMP	Membrane associated MMP

NCS	Neuronal calcium sensor
NO	Nitric oxide
P	Postnatal day
P1GF	Placental growth factor
PAK1	p21-activated kinase 1
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
PECAM	Platelet/endothelial cell adhesion molecule
PGI_2	Prostacyclin I_2
PI3K	Phosphoinositide kinase-3
PKC	Protein kinase C
PKG	cGMP dependent protein kinase
PLA_2	Prostacyclin A_2
PLC-γ	Phospholipase C-γ
PVD	Peripheral vascular disease
PyV-mT	Polyomavirus middle T
RBC	Red blood cell
RGD	Arginine-glycine-asparagine
RT-CES	Real-time cell electronic sensing
SDF-1	Stromal-derived factor-1
SIP1	Sphingosine-1-phosphate-1
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinase

TNFα	Tumor necrosis factor α
vWF	von Willebrand Factor
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VPF	Vascular permeability factor

CHAPTER 1

General Overview

1.1 The circulatory system

All except the most primitive animals must have a circulatory system in order to insure adequate distribution of essential elements necessary for survival. Even in organisms as diverse as mollusks, arthropods, and fish, the hierarchical branching of circulatory vessels and their inflowing blood facilitates the transport of nutrients, respiratory gases, hormones, metabolic products, and components of the immune system(1). In humans, the circulatory system is composed of a four-chambered heart that pumps blood to two fully separated circulations through the pulmonary and aortic trunks. The pulmonary trunk branches into the pulmonary arteries and carries blood to the lungs, while the aorta branches into the systemic arteries, which distribute blood to all other organs of the body(2). Within all perfused organs further branching of the blood vessels occurs, creating millions of tiny arteries that give rise to billions of downstream capillaries. These capillaries are microscopic in size, form vast networks, cover a large surface area, and connect the arterial and venous circulations (Figure 1-1). Remarkably, the most vital functions of the circulatory system occur in the microvasculature and are greatly dependent on the events that occur at the capillary level(3).

1.2 Microvascular capillaries

Microvascular capillaries are entirely responsible for the diffusion of water, small solutes, and lipid soluble molecules in the circulatory system(4). Furthermore, in the adult, microvascular capillaries are the site where new blood vessels are first formed to

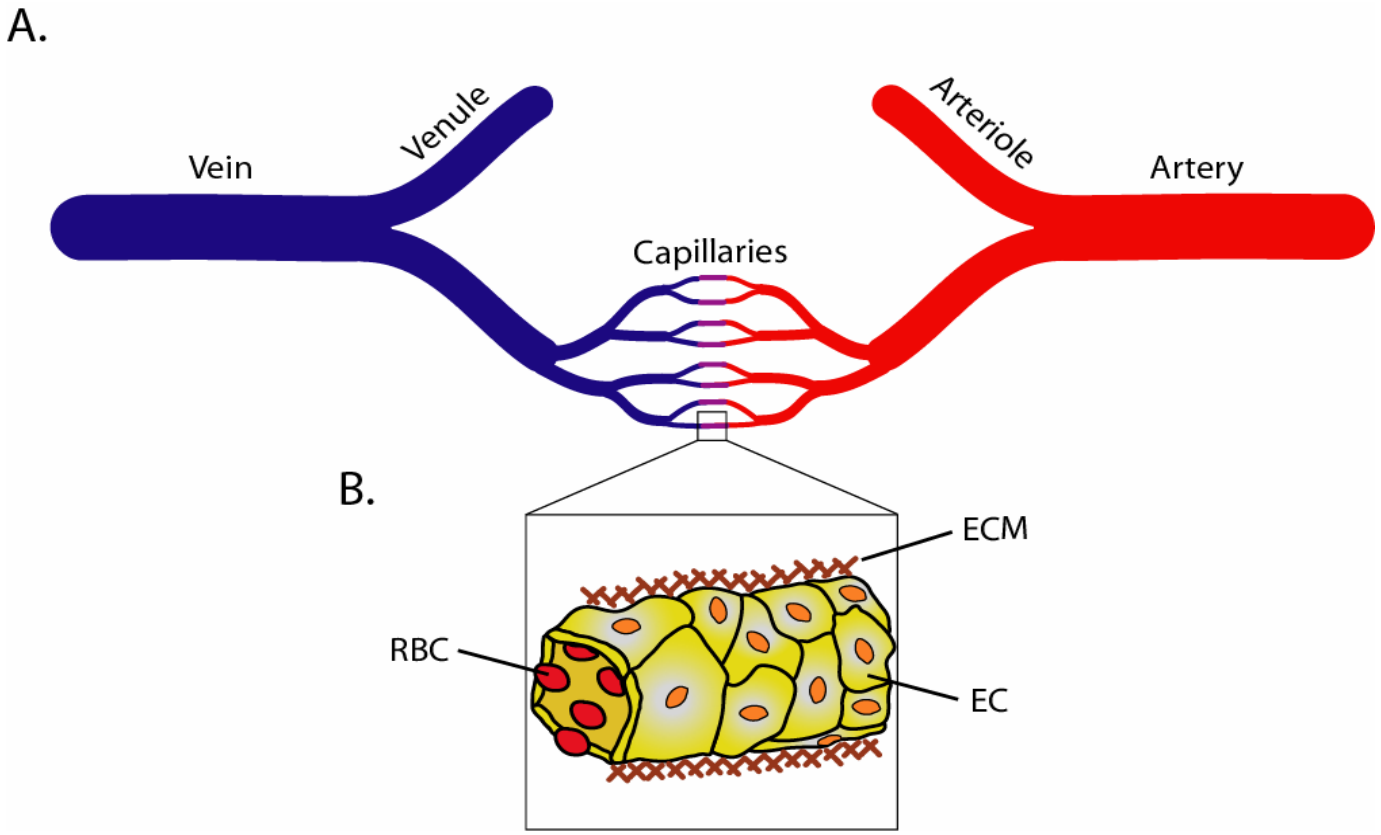

Figure 1-1. Capillaries are an essential component of the circulatory system. (A) The circulatory system is composed of an arterial and venous circulation. The hierarchical branching of vessels allows for proper distribution of blood and nutrients to all organs of the body. Major arteries stem from the heart and its conduit vessels and branch into arterioles. Arterioles are further partitioned to yield smaller vessels. Eventually they form single cell tubular capillaries and are primarily lined by ECs **(B)**. The structural integrity of these capillaries is also maintained by enveloping layers of ECM. Capillaries can coalesce into venous structures. These structures give rise to venules which then give rise to veins that facilitate the return of blood to the heart. EC, endothelial cell; ECM, extracellular matrix; RBC, red blood cell.

facilitate vascular expansion(5). Depending on the microenvironment, the vast majority of newly formed capillaries are referred to as continuous capillaries. These are structurally simple tubes, approximately 8μm in diameter, and are held together by cellular tight junctions(3). Continuous capillaries are also enveloped by a delicate layer of basement membrane composed primarily of extracellular matrix (ECM) proteins such as collagens, laminins, elastin, fibronectin, and vitronectin(6). This layer of ECM proteins is essential for maintaining the structural integrity of the microvascular capillary.

In addition to continuous capillaries, there are also fenestrated (or discontinuous) capillaries that contain pores that span the lining of the vessel and allow for a more rapid exchange of water and solutes(3). Fenestrated capillaries can occur physiologically and are naturally found in anatomical structures such as the brain choroids plexus and the kidney glomeruli. However, they are also a hallmark of the microvasculature in pathological tissue (such as in rapidly expanding tumors)(7). In either case, capillaries, both continuous and fenestrated, function as units of a dynamic vascular plexus that are highly sensitive to an array of local and external stimuli(8).

1.3 The endothelium

Capillaries are also almost entirely composed of endothelial cells (ECs), which are the cellular units that form the endothelium – the inner lining of all the vessels and the heart(4). Forming the interface between blood and tissue, at first glance the endothelium can be overlooked as a simple tissue that only serves an inert barrier function. However, with the

advent of *in vitro* primary culture of ECs, and more recently, with the use of genomic and proteomic techniques, ECs have been found to have a diverse topology and serve various complex functions(9;10). It is now accepted that in addition to its barrier function, the endothelium participates in various other functions including vascular permeability, vascular tone, trafficking of nutrients, leuckocyte recruitment and transmigration, inflammation, maintenance of blood fluidity, vascular morphogenesis, and vascular expansion (see Table 1-1 for a partial list of endothelial functions)(11). All these functions no doubt contribute to vascular homeostasis and are necessary for the survival and function of multiple types of tissue and organs.

1.4 ECs and angiogenesis

In the past few decades, several major breakthroughs were reached in our understanding of the signaling mechanisms that regulate ECs and their role in vascular expansion and new blood vessel growth. Much of this progress can be attributed to 'knockout' mouse models, where *in vivo* homologous recombination techniques are used to selectively delete genes and determine their purpose in the vasculature. As a result, knockout mouse models have helped to uncover a large number of essential genes that can regulate the endothelium and its various functions. One of these functions is angiogenesis, which is an EC-dependent process that is defined as the formation of new blood vessels from a pre-existing capillary network(12). Disregulation of angiogenesis can yield deleterious effects and can even be fatal in animal models(13-15). Furthermore, overstimulation and understimulation of angiogenesis are hallmarks of a myriad of clinical disorders(5;16;17).

Table 1-1. EC functions in vascular biology.

Function	Protein	Specific Role	Reference
Permeability barrier	PECAM1	Cell-to-cell adhesion	(18)
	VE-Cadherin	Cell-to-cell adhesion	(19)
	Integrin α2β1	Cell-to-cell adhesion and cell-to-matrix adhesion	(20;21)
Trafficking of nutrients	Caveolin 1	Forms trans-endothelial caveolae	(22)
Vasoactive factors	NO	Vasodilating agent	(23)
	adenine metabolites	Vasodilating agent	(24)
	endothelin	Vasoconstricting agent	(25)
Blood fluidity	PGI_2	Antithrombotic agent	(26)
	Factor VIIIa (vWF)	Prothrombotic agent	(27)
	thrombomodulin	Anticoagulant	(28)
	Tissue Factor	procoagulant	(29)
	Tissue plasminogen activator	Fibrinolytic agent production	(30)
Inflammation	IL-1	Expressed to mediates inflammation	(31)
Leukocyte recruitment	ICAM-1	Bind leukocytes and facilitates transmigration	(32;33)

Growth inhibition	Heparin	Inhibits EC proliferation	(34)
Growth stimulation	bFGF	Stimulates EC proliferation	(35)
	PDGF	Stimulates EC proliferation	(36;37)
Blood cell production	blood cell colony-stimulating factor	Regulates granulopoiesis	(38)

Abbreviations: PECAM1, platelet/endothelial cell adhesion molecule 1; VE-Cadherin, vascular endothelial cadherin; NO, nitric oxide; PGI_2, prostacyclin; vWF, von Willebrand Factor; ICAM, intracellular adhesion molecule 1; bFGF, basic fibroblast growth factor; PDGF, platelet derived growth factor.

Therefore, in recent years interest has heightened to exploit this process as means to treat patients with angiogenesis-dependent diseases.

1.5 The promise of angiogenesis-based therapy

It is predicted that over the coming decades more than 500 million patients will benefit from therapy that targets the endothelium to either inhibit or stimulate angiogenesis(39). As such, more than $4 billion has been invested in the research and development of novel angiogenesis-based medicines, making it one of the most highly funded areas of medical research today (The Angiogenesis Foundation; http://www.angio.org). To date, approximately 300 molecules that regulate angiogenesis have been discovered. However, only a subset of these molecules has progressed to clinical trials, and even a smaller subset has demonstrated clinical benefit in patients.

Nevertheless, the rate of progress in the development of new angiogenesis-based therapies has been very rapid. In a relatively short time, 7 angiogenesis-based medicines have been FDA approved to treat of a limited, yet diverse set of clinical disorders (http://www.angio.org). The recent success of some of these agents has prompted Phase IV clinical trials to determine whether these agents can also improve patient outcomes in other disease states. Similarly, results from Phase I and Phase II clinical trials testing new angiogenesis-based therapies are eagerly awaited (http://cancertrials.nci.nih.gov/). Hence, over the coming years it will be interesting to observe the enormous growth in this field and the emergence of angiogenesis-based medicines as agents of first-line therapy.

1.6 Goal of this research

The goal of this research is multifaceted. First, we wanted to expand our knowledge and understanding of the important regulatory protein calcium and integrin binding protein 1 (CIB1; see Chapter 3 for an overview of this protein). Despite its previously reported important functions in different cell types(40), its role in vasculature and in ECs has never been explored. Second, we wanted to determine whether CIB1 played a role in angiogenesis. We hypothesized that it would, since CIB1 was previously shown to regulate the activity of established angiogenic kinases(41;42). Finally, although CIB1-KO mice are viable and develop normally(43), we postulated that CIB1 may still have an important role in pathological and/or adaptive forms of angiogenesis. We tested this in various *in vivo* settings, and we report herein that CIB1 is involved in at least two forms of pathological angiogenesis (ischemia-induced angiogenesis and tumor-induced angiogenesis). We believe this research is substantial since it identifies a previously unknown role for CIB1, and since it delineates new differences between physiological and pathological forms of angiogenesis. By understanding these differences more thoroughly, a new generation of more selective and effective angiogenesis-based therapies can be developed to alleviate human disease.

1.7 Overview of thesis

Chapter 2 will briefly introduce the process of angiogenesis and its importance in human health and disease. This chapter will also summarize standard methods for modeling

and studying angiogenesis, as well as provide a non-exhaustive overview of essential molecular signals that regulate angiogenesis. Chapter 3 is an extension of the introduction and will primarily focus on CIB1 and its currently known roles in vascular biology.

In Chapter 4, data exploring the critical role of CIB1 in EC function and signaling will be presented. Specifically, the data will show that the loss of CIB1 in ECs leads to a decrease in migration, proliferation, nascent tubule formation, and monolayer permeability. We propose that these functional defects are at least in part due to the attenuated activation of p21-activated kinase 1 (PAK1) and ERK1/2 in CIB1-KO ECs. Furthermore, we show that CIB1 is essential for angiogenic growth factor-induced matrix metalloproteinease-2 (MMP2) expression, which ordinarily contributes to various pathophysiological conditions.

To expand these findings further, Chapter 5 will present data collected from two distinct *in vivo* murine ischemia-induced angiogenesis models. In summary, we demonstrate that CIB1 knockout (CIB1-KO) mice have a reduced capacity for pathological and adaptive neovascularization, despite demonstrating normal retinal vascular development. In this chapter, we also show that associated with decreased angiogenesis, CIB1-KO mice also have significantly reduced tissue recovery following ischemic insult.

In Chapter 6, we provide further evidence that implicates CIB1 in pathological angiogenesis. First we demonstrate that CIB1-KO tissue is stimulated less by angiogenic growth factors and as a result forms less microvessels *ex vivo* and *in vivo*. In addition, we explore the role of CIB1 in tumor-induced angiogenesis in two different xenograft tumor

models. Collectively these studies demonstrate that CIB1-KO mice develop tumors that are smaller in size, necrotic, and less vascularized. These observations are also corroborated by preliminary findings that demonstrate that CIB1-KO mice, that are also transgenic for the Polyomavirus middle T (PyV-mT) oncogene, have a sizably decreased tumor burden.

Finally, in Chapter 7, we conclude with a summary of the data presented in this thesis and highlight future experiments. We also make conclusions regarding the differences between physiological and pathological angiogenesis, and we speculate about the molecular signals that differentially regulate each process. We propose that targeting pathological forms of angiogenesis may yield better results in patients afflicted with angiogenesis-dependent diseases and suggest CIB1 as a novel target for anti- and pro-angiogenic therapy.

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CHAPTER 2

Role of Angiogenesis in Vascular Expansion

2.1 The basic model of angiogenesis

Angiogenesis is a dynamic multi-step process that is highly dependent on EC signaling and function, and is defined as the formation of new blood vessels from pre-existing vascular networks(1;2). In addition to contributing to the expansion of the primitive vasculature in the embryo, angiogenesis is the primary mechanism by which most new blood vessels are formed in adult tissue during both physiological and pathological situations(3). Thus understanding the mechanisms that can inhibit or enhance angiogenesis may have significant implications for human health and disease.

Under normal conditions, in adult mammalian tissue the endothelium (which is derived from embryonic hemangioblasts) is thought to be quiescent and angiogenesis is assumed to be static. In fact, in the normal adult it is estimated that at any given time only 1 in every 10,000 ECs is undergoing cell division(4). However, in response to appropriate stimuli, quiescent ECs become activated and facilitate the growth of new capillaries, or regress and undergo apoptosis(5;6). Thus EC regulation is complex and often multifaceted due to the various physiological and pathological stimuli, cytokines, hormones, and growth factors that can determine the activation state of ECs(7;8). The delectate balance between the factors that activate ECs and stimulate angiogenesis (pro-angiogenic factors), and the factors that halt ECs and inhibit angiogenesis (anti-angiogenic or angiostatic factors) is often referred to as the 'angiogenic switch(4).' This basic model suggests that when angiogenic growth factors in the EC microenvironment surpass a specific threshold, angiogenesis will

proceed. On the other hand, if angiostatic agents dominate, or cell-cell and cell-matrix interactions are not in place, ECs will be inhibited and angiogenesis will not occur(9).

In 1984, basic-fibroblast growth factor (bFGF; also known as FGF-2) became the first angiogenic growth factor to be discovered(10;11). Since then, many such growth factors have been identified, including vascular endothelial growth factor (VEGF), placental growth factor (P1GF), PDGF, and transforming growth factor-β (TGF-β) (for a partial list of endogenously expressed pro- and anti-angiogenic factors see Table 2-1)(12-14). These growth factors have the capacity to activate vascular ECs via their respective cell-surface receptors. Upon EC activation, vessel permeability is increased, the secretion of proteolytic enzymes is induced, and the degradation of surrounding extracellular matrix occurs. This process facilitates the release of additional growth factors from the ECM and stimulates EC proliferation(15). Activated ECs also respond to gradients of growth factors in their environment through the extension of cellular processes (filopodia and lamellipodia)(16;17). These extensions allow EC sprouting to take place, which then determines the directionality of EC chemotatic migration(17;18). Similarly, activated ECs can also engage in haptotatic migration, which is migration toward ECM proteins such as fibronectin, collagen, and vitronectin. This process of haptotaxis requires the expression of specific adhesion receptors, such as integrins, on the surface of ECs(19;20). For both chemotatic and haptotatic migration, the trail set forth by sprouting ECs establishes the location of future capillaries. At these sites EC invade and implant into the ECM. This allows them to eventually envelop one another and form lumens of what later becomes nascent tubules (Figure 2-1).

Table 2-1. Examples of endogenously expressed pro- and anti-angiogenic factors.

Etiology	Protein	Reference
Pro-angiogenic Factors:		
Growth factors	VEGF-A, -B, -C, -D	(21)
	aFGF, bFGF	(22)
	P1GF	(23)
	PDGF	(24)
	TGFα, TGFβ	(25)
	IGF-I, -II	(26)
	HGF	(27)
Hormones	Estrogens	(28)
	Leptin	(29)
	hCG	(30)
	proliferin	(31)
Inflammatory cytokines	IL-8	(32)
Anti-angiogenic factors:		
Secreted peptides	Thrombospondin	(33)
	Endostatin	(34)
	Angiostatin	(35)
Hormones	Prolactin	(36)

Abbreviations: VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; P1GF, placental growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; IGF, insulin growth factor; HGF, hepatocyte growth factor; hCG, human chorionic gonadotropin.

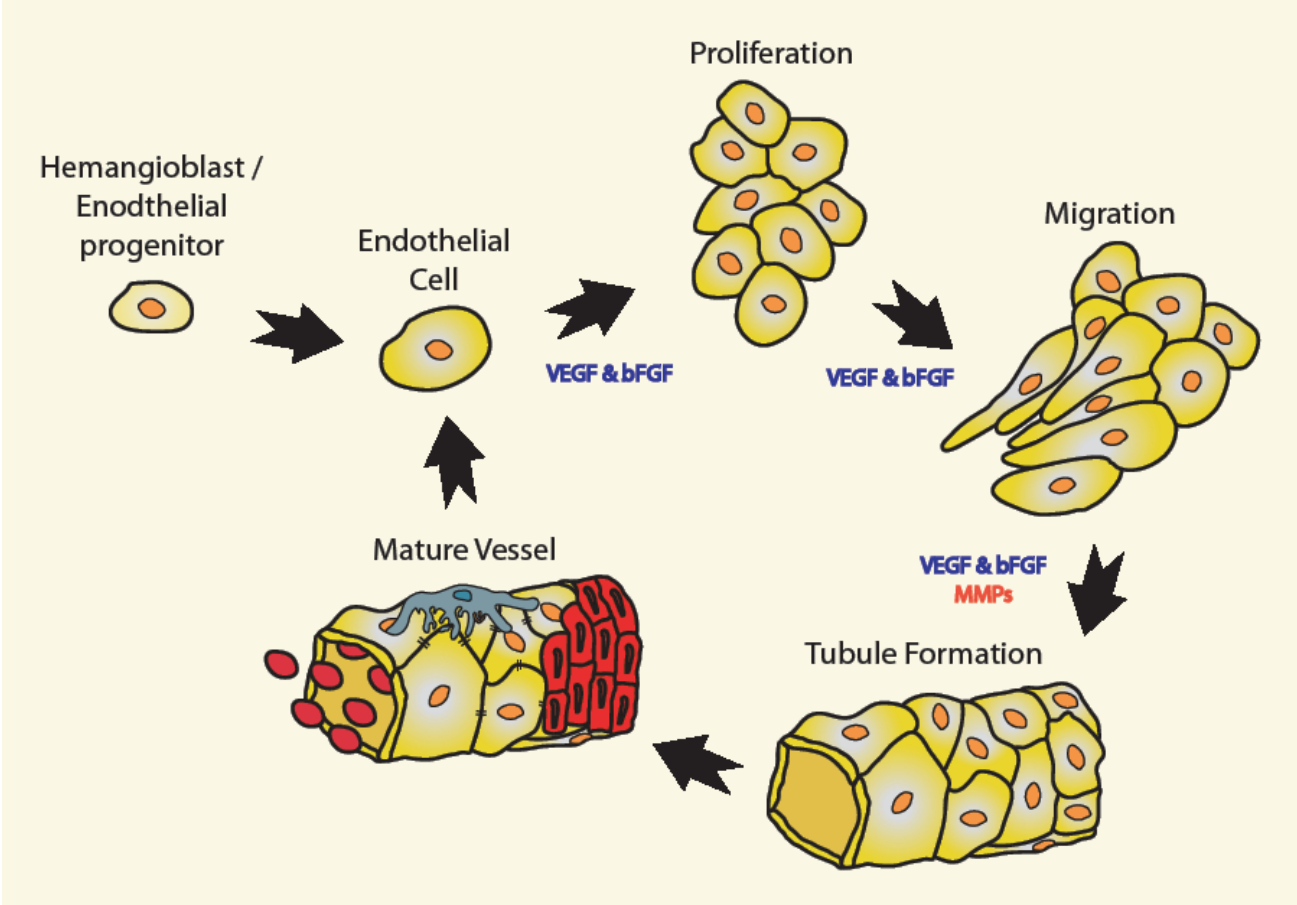

Figure 2-1. Angiogenesis is a multi-step process. ECs that are derived from mature vessels, or hemangioblasts/endothelial progenitor cells, can undergo activation by angiogenic growth factors such as VEGF and bFGF. Upon activation, ECs undergo a remarkable increase in proliferation, directed migration, and tubule formation. These events characterize the activation phase of angiogenesis, which is regulated by growth factors and matrix metalloproteinases (MMPs). During the resolution phase of angiogenesis, these newly formed tubules undergo remodeling and recruit other vascular cells such as pericytes and smooth muscle cells.

In the resolution phase of angiogenesis, newly formed nascent tubules undergo stabilization, maturation, and remodeling to become actual capillaries(37). This process is facilitated by the recruitment of mesenchymal cells and is highly dependent on the origin, location, and function of the newly formed capillary. Studies have also shown that molecules such as PDGF-β, PDGF-β receptor (PDGFR)-β, sphingosine-1-phosphate-1 (SIP1), and endothelial differentation sphingolipid G-protein-coupled receptor-1 (EDG1) are essential components to this process(38;39). Molecules such as these also facilitate new vessel branching, expansion, and pruning in order to meet local tissue demands. Finally, surrounding mesenchymal cells differentiate to give rise to smooth muscle cells, pericytes, and fibroblasts which, in turn secret additional ECM proteins and facilitate further stabilization of the maturing newly formed capillaries(37).

2.2 Other mechanisms of vascular expansion

2.2.1 Vasculogenesis

One of the most important ECs functions is the formation of new blood vessels during embryogenesis(40). In the embryo the circulatory system develops shortly after gastrulation and is the first functioning organ system(41). At this stage the vascular primordia forms clusters of progenitor cells, known as hemangioblasts, and arise in blood islands in the yolk sac between the embryonic mesoderm and endoderm(42). Through a programmed sequence of events these hemangioblasts differentiate *de novo* into peripheral cells, which become either angioblast ECs or short-lived primitive hematopoetic stem cells (HSCs)(43;44). Due

to their lineage association, these newly formed and spatially segregated ECs and HSCs share several surface expression markers including the VEGF receptor 2 (VEGFR-2; Flk-1), TEK tyrosine kinase (Tie2), and CD34(45;46). Further differentiation of these hemangioblast-derived cell types marks the beginning of vasculogenesis and is a highly conserved process.

Unlike angiogenesis, vasculogenesis facilitates vascular expansion *de novo* (for a comparison between angiogenesis and vasculogenesis see Table 2-2)(47). As embryonic development progresses, newly formed ECs in the visceral yolk sac rapidly divide and form a primitive vascular plexus. This plexus coalesces with a system of EC tubules that originates independently within the parenchyma of the embryo(48). At this stage ECs start to assume their primary barrier function by maintaining the interface between blood and tissue. Remarkably, the primitive vascular plexus derived by vasculogenesis is highly ordered, with channels having the same width and separated by approximately similar distances(40). Therefore, it is not surprising that any disruption of this highly ordered system often results in death by embryonic day (ED) 9.5 to ED 12.5 in mouse embryos(49). For instance, gene deletion of molecules that facilitate VEGF and Notch signaling cause death in ED 9.5 - 10.5 mouse embryos(50). These findings, in various other murine gene knockout studies, have been instrumental in the identification of genes that are important for proper vessel development and remodeling.

In some circumstances post-natal vasculogenesis has been suggested to also occur in the adult(51). One of the first studies to suggest this demonstrated that endothelialization of prosthetic devices in adult canines can occur via differentiation of circulating endothelial

Table 2-2. General comparison between vasculogenesis and angiogenesis.

	Vasculogenesis	Angiogenesis
Method of new blood vessel formation	*De novo*	From pre-existing capillaries
Depends on what cell types	Progenitor cells (hemangiocytes/angioblasts)	Mature ECs in capillary bed
Occurs in adult vs. embryo	Primarily in the embryo	In the embryo and adult
Regulation	Highly ordered process	Not as tightly regulated, especially during pathological conditions
If impaired	Frequently leads to lethality	Deceased wound healing, ischemia, and vascular insufficiency
If excessive	Frequently leads to lethality	Causes vascular malformations, contributes to tumor growth, and facilitates inflammation

progenitor cells (EPCs) that are CD34-positive(52). Similarly, Asahara et al. showed that adult circulating EPCs can differentiate into mature ECs *in vitro* and contribute to ischemia-induced new blood vessel formation *in vivo*(53). Other investigators also showed that human cord blood EPCs also have clear vasculogenic properties following hind-limb ischemia(54). Thus the contribution of EPCs in adult vasculogenesis is becoming a more commonly accepted concept.

2.2.2 Circulating progenitor ECs

In addition to their contributions in post-natal vasculogenesis, EPCs also contribute to other forms of neovascularization, particularly in the context of rapidly growing tumors(55;56). One hypothesized source of EPCs is from the bone marrow. *In vitro*, EPCs derived from the bone marrow can be enriched and differentiated into ECs(57;58). *In vivo*, bone marrow-derived ECs were found at sites of neovascularization in athymic nude mice that were transplanted with bone marrow derived from transgenic mice constitutively expressing β-galactosidase via an endothelial cell-specific promoter (Tie2/lacZ)(59). Additional studies confirmed the presence of bone marrow-derived ECs at the sites of neovascularization post-myocardial ischemia, hind-limb ischemia, wound healing, atherosclerosis, and retinal neovascularization(51). However, despite agreement on their presence, some reports claim up to 95% of the neovascularization can be attributed to bone marrow-derived ECs(60), but other studies report contributions ranging between 3.5 to 10%(61). Thus, it is still debated to what extent bone marrow-derived ECs can contribute to the formation of new blood vessels.

Like the bone marrow, hematopoietic stem cells (HSCs) are also hypothesized to yield ECs and contribute to neovascularization(51). This was originally hypothesized since ECs and hematopoietic cells have a close lineage in the blood islands of the developing embryo. Moreover, hematopoietic cells and ECs are derived from a common hemangiocyte progenitor cell. Evidence to support this comes from findings that show that both hematopoietic cells and ECs express common putative markers such as Flk-1, Tie2, and CD34(62;63). Furthermore, Jin et al. recently showed that cytokine release of stromal-derived factor 1 (SDF-1) can mobilize to sites of neovascularization a population of non-endothelial hemangiocytes that express CXCR4 (the receptor for SDF-1) and VEGFR-1(64).

Additionally, contributions from endothelial-derived circulating cells (EDCCs) in vascular expansion have also been reported. It is hypothesized that these cells result from a shedding process involving the delamination of ECs from pre-existing vasculature, or generated as a result of injury or mechanical disruption(51). However, Segal et al. recently reported that the number of EDCCs observed in the blood stream following myocardial infarctions cannot account for total EDCC levels(65). Furthermore EDCCs are found in the circulation even under normal physiologic conditions. Thus it is not entirely clear how EDCCs are generated and whether their fate is exclusively restricted to ECs(51).

2.2.3 Non-sprouting angiogenesis

In addition to sprouting angiogenesis, non-sprouting angiogenesis can also form new blood vessels from pre-existing vessels. One such process is referred to as

intussusception(66). This mechanism of vascular growth is somewhat unique because it occurs through the formation of intra-vascular transendothelial cell bridges, whereby interstitial ECs form columns within in vessels and partition the vessel lumen. Circulating ECs (i.e. EPCs and EDCCs) are postulated to contribute to this process. Reinforcement of the newly formed EC bridges occurs by the recruitment of smooth muscle cells and pericytes. Remodeling of the newly formed bridges also takes place to stabilize the newly formed lumens and leads to vessel maturation(67). Although it is reported that this type of vascular expansion frequently occurs in rapidly growing tumor vasculature(68), little is actually known about the frequency, extent, and molecular basis of intussusception *in vivo*.

2.2.4 Arteriogenesis and mechanical-induced forces

It is well documented that blood vessels have the capacity to adapt to altered flow situations(69). Arteriogenesis describes this process and is defined as the remodeling of pre-existing collaterals between arteries of adjacent vascular beds(70). Unlike angiogenesis, which is regulated by hypoxia and angiogenic growth factors, arteriogenesis is instead primarily regulated by physical forces that occur in and around the vasculature(71). In fact, *in vivo* fluid sheer stress is known to be one of the strongest experimental triggers for arteriogenesis(72). In such models, shear stress in the collaterals is increased upon stenosis, occlusion, or ligation of conduit arteries, and is transmitted to endothelial, stromal, and mesenchymal cells on a molecular and cellular level. For example, stretch of ECs may be sensed by integrins that are anchored to ECM, as well as tyrosine kinases and ion channels on the EC surface(73). These effects can induce rearrangements of the cytoskeleton and

induce MAP kinase (MAPK) signaling, which in turn transmit signals to the nucleus(74). To date, a large number of genes have been reported to be transcriptionally up-regulated by promoters containing shear stress responsive elements(71). Among those reported to be increased, is the expression and secretion of plasminogen activators and MMPs. These factors degrade and remodel the ECM, therefore enabling cell proliferation and migration(15). Thus mechanical forces, including fluid shear stress, can induce complex cascades of molecular and cellular events that lead to larger vessel lumens and increased wall thickness.

2.3 Physiological angiogenesis

2.3.1 Wound healing

In addition to its role in the development of the embryo, physiological angiogenesis is also important for rare events in the adult. For example, physiological angiogenesis plays an important role in healing, which is essential for the recovery from fractures or wound injuries(3). It is not only critical for repair, but it also restores tissue strength and is necessary for resistance from infection and further external insults.

The process of wound healing is based on three phases of healing(75). First, inflammatory cells migrate into the wound. This is called the lag phase and it occurs over the span of several of days following the injury. Neutrophils predominate in the wound for approximately 1-2 days, and then macrophages become active by approximately the third

day. The abundance of inflammatory cells leads to the secretion of various cytokines and inflammatory mediators forming a significant chemoattractant gradient in the surrounding tissue. This positive gradient induces the next phase of wound healing, which is called the proliferative phase. During this 4-5 week phase, fibroblasts and ECs migrate into the wound, fibroblasts lay down a collagen matrix, and angiogenic ECs form nascent capillary loops from the adjacent venules at the wound margin. The newly formed capillary buds are highly permeable and grow across the wound to supply the fibroblasts with oxygen and nutrients. Both of the capillaries and the surrounding fibroblasts are critical components for facilitating tissue binding and ultimately wound strength. Evidence for this comes from patients with Vitamin C deficiencies, who have less efficient synthesis and cross-linking of collagen, which as a result impedes capillary formation and neovascularization of a wound(76).

2.3.2 Reproduction and pregnancy

The cyclic development of new vasculature plays a critical role in the reproductive tract of all mammalian females and is involved in endometrial growth, embryo implantation, and placentation(14). In the ovary, follicular growth, and subsequently, during ovulation and formation of the corpus luteum, a vascular network emerges within the ovarian thecal cell layer(77). Similarly, the endometrial vasculature in humans undergoes dramatic cyclic changes, and in both the proliferative and secretory phases of the menstrual cycle, the endometrium undergoes dramatic vascularization(78;79). This relatively rapid transformation in endometrial vasculature is characterized by an increase in length, branching and coiling of the uterine spiral arteries. The changes observed in both the ovary and uterus

are highly regulated by the actions of several angiogenic growth factors and hormones. For example, VEGF message levels increase almost three-fold during the secretory phase of the menstrual cycle(80). Similarly, angiopoitein 1, and 2 (Ang1 and 2) secretion is increased up until menstruation and then decreases during the proliferative phase of the menstrual cycle(81).

Physiological changes in the uterine circulation are also hormonally regulated. For example, recent studies have shown that the ovarian hormones progesterone and estrogen can induce significantly uterine stromal cell expression of VEGF, and progesterone can upregulate endometrial bFGF secretion(82;83). Similarly, uterine angiogenesis can also be induced by hCG, a hormone secreted by the corpus luteum that is normally responsible for maintaining pregnancy-induced changes and the formation of the placenta. It is suggested that hCG mediates its effects by activating protein kinase C (PKC) and MAPK (ERK1/2) signaling(14). Independently, studies also demonstrate that hCG can induce angiogenesis by stimulating the migration and capillary sprouting of uterine ECs(84).

During pregnancy, an adequate nutrient and substrate supply is essential for the normal growth and development of an intrauterine fetus. In order for such fetal demands to be met, the uterine vasculature must adapt accordingly. The endometrium, uterine decidua, and placenta are rich sources of angiogenic growth factors like VEGF, bFGF, and PIGF, which collectively induce dramatic changes in the endometrium(14). First, vasodilation is stimulated to increase the blood volume that participates in the process of fetal gas exchange(77). Second, pre-existing maternal uterine vessels become more permeabiable,

thus allowing for more rapid nutrient/waste exchange(78). Lastly, angiogenesis is stimulated by the signaling mechanisms initiated by activated growth factor receptor tyrosine kianses(85).

This process of new blood vessel formation is especially important at the earliest stages of pregnancy (7-11 days post conceptionem). During early pregnancy intracellular signals propagated from activated growth factor receptors induce proliferation, chemotatic migration, and invasion of ECs into the endometrium. As a result intact capillaries grow around the fertilized ovum syncytiotrophoblast layer(78). These newly formed capillaries form a plexus that then connects into the emerging placenta and give rise to a primitive vascular system that supplies the embryo. Profound changes in vascular patterning occur deeper in the uterine tissue, yielding permanent changes even following pregnancy(14). Due to its complexity, it is still not entirely clear, how the level of angiogenesis in the endometrium and placenta is tightly regulated throughout pregnancy to prevent both over- and under-stimulation of new blood vessel growth – which in either case would be detrimental to both the mother and fetus.

2.4 Pathological Angiogenesis

2.4.1 Ischemic diseases

In contrast to physiological angiogenesis, pathological angiogenesis underlies the pathophysiology of a myriad of clinical diseases, which include ischemia-related clinical

disorders(3;86). Upon induction of ischemia, tissues become metabolically stressed due to decreased nutrient and oxygen levels, and increased waste levels. Eventually this leads to hypoxia and activation of the hypoxia inducible factor-1 (HIF-1) transcription factor, which induces the expression of various angiogenesis-related growth factors and their receptors(86). These growth factors in turn facilitate an adaptive angiogenic response in the ischemic tissue as an attempt to restore proper blood perfusion. Often however, this rapid induction of angiogenesis can be excessive and as a result can lead to aberrant blood vessel growth and permanent tissue damage. This type of ischemia-induced pathological angiogenesis is commonly observed in different types of retinopathies, including diabetes mellitus retinopathy, central retina vein occlusion-induced retinopathy, and retinopathy of prematurity(87-90). In each one of these conditions retinal neovascularization is in part stimulated by the upregulation of hypoxia-induced VEGF. In agreement with this, elevated VEGF levels have been detected in both the aqueous and vitreous fluids of patients with proliferative and non-proliferative forms of retinopathy(91). Rodent models have also established the role of VEGF signaling in ischemia-induced retinopathy of prematurity(89;92).

The goal for patients afflicted with retinopathies is to inhibit ischemia-induced pathological angiogenesis as much as possible. However, in patients afflicted with ischemic heart disease and peripheral vascular disease, the induction of ischemia-induced angiogenesis can be of great clinical benefit for alleviating disease symptoms(93). This field of study is obviously immensely important since each year in the United States diseases of the heart are reported to account for 30% of all deaths (http://www.americanheart.org), and an estimated

7.4 million people develop critical limb ischemia(86). Surgical revascularization techniques and pharmacological treatments are available to treat these conditions; however they only benefit a subset of patients. Thus, for the majority of patients suffering from ischemic diseases, therapeutic angiogenesis may be of great clinical benefit.

2.4.2 Genetic diseases

Vascular deformities and anomalies may have an inherited predisposition, often resulting from genetic mutations in cellular and molecular components that regulate ECs and smooth muscle cells(94). Hemangiomas are vascular tumors and are common vascular deformities that are angiogenesis dependent(95). The most common type of hemangioma is infantile hemangioma and it is characterized by increased bFGF and Tie2 expression, enhanced response to Ang1, and disregulated Ang2 expression(96). In addition to arising from somatic mutations in angioblasts during development, infantile hemangiomas are also hypothesized to possibly be derived from immobilized placental vascular cells that become dislodged into the fetal circulation during gestation or after birth(97).

Many mutations have also been identified in different genes that encode essential angiogenic products. Often these mutations are found to lead to severe clinical conditions. For example, mutations in the Tie2 receptor gene at locus 9p21, leads to an autosomal dominant condition known as coetaneous-mucosal venous malformation that is characterized by decreased smooth muscle cell recruitment to the venous circulation(98;99). Mutations in endoglin and ALK1, which are critical components of TGF-β signaling, predispose patients

to develop arteriovenous malformations that manifest in syndromes that are clinically referred to as hereditary hemorrhagic telengiectasia (HHT)-1 and HHT-2(97). Mutations also in Notch3, a gene implicated in interactions between ECs and smooth muscle cells, leads to a condition called cerebral autosomal dominant arteriophaty with subcortical infarcts and leucoencephalopathy (CADASIL)(100).

2.4.3 Pregnancy associated disorders

Vascularization of the human embryo takes place very early in pregnancy and depends highly on proper vascularization of the maternal placental chorionic villi(14). If placental chorionic villus varscularization is even slightly compromised, conditions such as preeclampsia, eclampsia, and intrauterine embryonic death can occur(101). In these events, chorionic villi were found to have a significantly reduced vascular density, constricted vessels, increased fibrosis, and hydorpic degradation. Recently, it was also reported that the decidual endothelium of placentas associated with idiopathic spontaneous abortions had diminished placental trophoblastic VEGF immunoreactivity, reduced placental torphoblastic Tie1 and Tie2 expression, and reduced VEGFR-1, VEGFR-2, Tie1 and Tie2 expression(102). Therefore, a growing body of evidence suggests that reduced placental blood vessel density is associated with poor pregnancy outcomes and likely has an important role in maternal fetal health.

2.4.4 Inflammatory diseases

Prolonged and excessive angiogenesis is a characteristic of various common inflammatory disorders(68;93). This effect is mediated by tissue macrophages and blood-borne monocytes, platelets, mast cells, and other leukocytes that release a myriad of angiogenesis factors such as VEGF, Ang1, bFGF, TGF-β, PDGF-β, IGF-1, tumor necrosis factor α (TNFα), HGF, and monocyte chemoattactant protein-1 (MCP-1)(103;104). These growth factors in turn activate ECs, stromal cells, and tissue resident macrophages, which secrete ECM proteinases such as MMP2, MMP7, MMP9, and MMP12(105). This process leads to degradation of anatomical barriers and facilitates neovascularization. Moreover, inflammatory cytokines such as TNFα also upregulate EC expression of adhesion molecules such as ICAM-1 and VCAM-1, which promote further inflammation-induced angiogenesis by promoting ECs and leukocytes interactions.

One of the most common inflammatory conditions is rheumatoid arthritis, which is a chronic systemic inflammatory disease of unknown etiology that occurs worldwide with a prevalence of 1%(106). In this condition, the number of tissue macrophages that are derived from CD34 positive bone marrow progenitors is increased in synovial membranes and pannus of joints(93). The activation of macrophages (which is demonstrated by the macrophage surface overexpression of major histocompatilibity complex class II molecules) induces secretion of growth factors, MMPs, cytokines, and chemokines that alter the normal balance between pro- and anti-angiogenesis factors in the synovium and stimulate neovascularization(107).

Psoriasis is another chronic inflammatory disorder that affects 2-3% of the Caucasian population, and is characterized by aberrant angiogenesis in the skin(108). Forty percent of psoriasis patients also have inflammatory arthritis (psoriatic arthritis), which form the second most common diagnostic category after rheumatoid arthritis in synovitis clinics(109). Although it is accepted that this condition develops due to abnormal activation of T cells and dentritic cells, as well as the abnormal secretion of inflammatory cytokines, the molecular alterations that underlie this disease are still not fully understood. Recently, genetically modified mice have revealed additional clues and have demonstrated that disregulated blood vessel growth contributes to the progression and severity of this disease(110). For example, when VEGF is overexpressed in the basal layer of the epidermis, mice develop psoriatic skin lesions that are hyperplastic, inflammed, and have epidermal thickening (110;111).

2.4.5 Oncogenic diseases

Pathological angiogenesis is a hallmark of tumorgenesis and is a discrete event in solid tumor formation(4;68). The observation that angiogenesis occurs around tumors was made nearly 100 years ago, and later, diffusible substances were hypothesized to emerge from a tumor to stimulate the growth of surrounding blood vessels(112). Now that many of these factors have been identified, it is widely accepted that various metabolic and mechanical stresses in tumors indeed induce the secretion of many factors and cause an imbalance in the so called angiogenic switch, to favor angiogenesis(4). Some of these metabolic stresses such as low pO_2, low pH, and hypoglycemia are induced when tumors

grow beyond $1cm^3$ and become hypoxic(113). These events activate key transcription factors, such as the heterodimeric transcription factor HIF-1, which can induce expression of a vast array of gene products (almost 2% of all human genes) to control energy metabolism, survival, and angiogenesis. Among the most highly regulated genes by HIF-1 are the genes encoding VEGF, VEGFR-1, and the glucose transporter GLUT-1(68).

Unlike physiological angiogenesis, the pathological induction of angiogenesis in tumors is temporally and spatially disregulated. As such, newly formed tumor vasculature is structurally and functionally abnormal. In contrast to normal vessels, tumor vessels are highly disorganized, tortuous, dilated, and have excessive branching and shunts(68). The endothelium that lines tumor vessels is also abnormal. Tumor ECs assume a different morphology, with absent or widened inter-endothelial junctions that sometimes project into vessel lumens(114). Some tumors vessels are not even lined with ECs, and are instead mosaic in nature and lined primarily with cancer cells that mimic the role of ECs. Vessel wall diameters are also uneven, have numerous openings and fenestrations, and often have a discontinuous basement membrane(115). Consequently, the heterogeneity and leakiness in vasculature leads to highly chaotic and variable blood perfusion within the tumor. This induces further hypoxia and acidosis, which in turn induces additional vessel and tumor growth(68;115). As a result tumor-induced angiogenesis facilitates a self-perpetuating vicious cycle of tumor growth that can ultimately lead to cancer metastasis. For this reason it is hypothesized that therapeutic targeting of tumor-induced angiogenesis may prevent progression of disease and diminish tumor growth.

Beyond solid tumor growth, disregulated angiogenesis is also observed in haematological malignancies and hemangiomas(68). For example, the relatively simple bone marrow vasculature in normal individuals is altered with complex branching in leukemic patients(116). Evidence suggests that malignant haematopoietic cells express and secrete factors like VEGF and Ang1, which induce these alterations, and perpetuate the progression of disease in the bone marrow(117). Similarly, VEGF signaling has been implicated in hemangioma growth, which is induced by genetic alterations (as mentioned earlier under genetic diseases) or viral infections. Human immunodeficiency virus type-1 (HIV-1) activates VEGFR-2, α5β1, and αvβ3 to give rise to Kaposi's sarcoma, a type of malignant hemangioma(118). Since these types of cancers are also dependent on angiogenesis, it is currently being determined whether anti-angiogenic therapy may be a useful adjunct to reduce the mortality and morbidity associated with these conditions.

2.5 Commonly used tools to study angiogenesis

2.5.1 *In vitro* assays

After approximately three decades of studies, investigators in the fields of vascular biology, and its major branch of angiogenesis research, have derived reliable and standardized methods that can be used to assess EC function and angiogenesis(119). Perhaps the most commonly used techniques are *in vitro* methods that can assess EC signaling and function. These techniques are low cost, reproducible, and simple compared to other angiogenesis assays. However, central to all *in vitro* assays is the ability to isolate and

culture ECs. This process requires a careful assessment of what EC cell-type should be isolated and how they should be cultured. As demonstrated in the literature, not all ECs are alike and differences do exist between macrovascular and microvascular ECs, EC obtained from different organs, and ECs isolated from different sites within a single organ(120). In addition, ECs used *in vitro* are by the very nature of being cultured, different from ECs that comprise the quiescent endothelium in the mammalian vasculature. To this effect, cultured ECs can both gain and lose attributes found in cells *in vivo*(121). Nevertheless, *in vitro* techniques have been instrumental for screening for anti-angiogenic factors and for testing various *in vitro* EC functions such as migration, two-dimensional and three-dimensional tubule formation, growth, proliferation, permeability, sprouting, and invasion.

2.5.2 *Ex vivo* assays

Angiogenesis involves not only ECs, but also other vascular cell types, including smooth muscle cells, fibroblasts, pericytes, and other mesynchemal cell types. This has led investigators to develop techniques that can readily assess angiogenesis in whole tissue. The rat and mouse aortic ring organ culture system is the most commonly used technique used for this purpose(122). This technique has also become more popular with the discovery of Matrigel (a matrix-rich product prepared from Engelbreth-Holm-Swarm (EHS) tumor cells whose primary component is laminin(123)), which can facilitate the growth and extension of microvessels from whole tissue organ cultures. However, one disadvantage of an aortic ring organ culture assay is that forms microvessels that are macrovascular in nature(120). Since angiogenesis is primarily a microvascular event, other tissue (such as hind-limb muscle

tissue) which contains microvessels can also be used to corroborate findings from aortic ring organ cultures.

2.5.3 *In vivo* assays

Depending on what is being investigated and what the experimental design entails, *in vivo*, *ex vivo*, and *in vitro* techniques can be used to complement one another. However, due to the cellular and molecular complexities of angiogenic interactions, *in vivo* studies are generally accepted to be the most informative(120). Indeed, in the preclinical setting, *in vivo* angiogenesis assays present the investigator with unique advantages to assess angiogenic processes in response to various types of stimuli, treatments, or trauma that are both physical or chemical in nature. Meanwhile, *in vivo* techniques also have some biological, technical, and economical limitations associated with them and therefore are not appropriate for all situations.

For evaluating both pro- and anti-angiogenic responses, *in vivo* assays such as the rodent corneal micropocket, the rodent mesentery, the chick chorioallantoic membrane, and the zebrafish system have been commonly used(124). Similarly, neovascularization of artificial implants can also be tested *in vivo* by implanting rodents with subcutaneous Matrigel plug impregnated with tumor cells or angiogenic agents. In such assays the extent of new blood vessel formation in a non-cellular matrix can be highly formative(124). Furthermore, *in vivo* assays can also be modified to test specific forms of pathological or adaptive angiogenesis. For example, ischemia can be induced in a rodent retina or hind-limb

muscle tissue to investigate processes that contribute to post-ischemic recovery and ischemia-induced pathological angiogenesis(125). Using the same paradigm, tumor- and inflammation-induced angiogenesis can also be assessed *in vivo*(119).

2.6 Molecular regulation of angiogenesis

In 1971, Folkman proposed a model whereby tumor growth and metastasis are angiogenesis-dependent(126). Hence, it was hypothesized that blocking angiogenesis could be an effective strategy for arresting tumor growth. Since then the field of angiogenic signaling has become an intense area of study. In recent years, our understanding of how new blood vessels develop during physiological and pathological situations has been significantly enhanced, and much of this progress could be attributed to homologous recombination techniques in murine mouse models. A partial list of 'knockout' mouse models used over the past two decades to study angiogenesis *in vivo* is provided in Table 2-3. As demonstrated in this table, the deletions of a significant subset of genes do not yield lethal phenotypes. This is despite the fact that some of these genes contribute to adult and pathological forms angiogenesis. Hence, this demonstrates that angiogenic signaling is complex and is differentially regulated by multiple molecular mechanisms.

In general angiogenic signaling molecules can be sub-divided into six main categories: i) growth factors and their receptors, ii) integrins, cadherins, and other cell surface adhesion receptors, iii) matrix degrading proteineases, iv) chemokines, hormones, and small

Table 2-3. A partial list of vasculogenesis and angiogenesis-related knockout mouse models.

Classification	Protein	Lethal	Description	Reference
Matrix Metalloproteniases (MMPs)				
	MMP2	No	Mild phenotype with minor defects in the skeleton and joints. Tumor-induced angiogenesis and tumor growth is highly reduced.	(133;134)
	MMP9	No	Show delayed visualization and ossification of hypertrophic zones in cartilage. Have decreased atherosclerosis and less aneurysm formation. Otherwise viable with only minor defects.	(135-137)
	MT1-MMP	No	Have severe skeletal abnormalities and do not mature sexually. Also have abnormal lung alveolar formation and decreased alveolar surface area.	(138;139)
	MMP2 & MT1-MMP	Yes	Death occurs immediately after birth due to respiratory failure, abnormal blood vessels, and immature muscle fibers.	(140)
	MMP2 & MMP9	No	Mice are normal but one report suggests a defect in transepithelial chemokine gradient formation.	(141;142)
	TIMP1	No	Has increased susceptibility to atherosclerosis-induced aneurysm formation.	(143;144)
	TIMP2	No	Has impaired proMMP2 activation and altered nerve branching.	(145;146)
	TIMP3	No	Has impaired lung development and dilated cardiomyopathy	(147;148)
Growth Factors				
	PDGF-β	Yes	Embryonic lethality due to lack of	(149;150)

		pericytes recruitment which causes the formation of microvascular aneurysms.	
Edg1	Yes	Like PDGF-β mice. Embryonic lethality due to lack of pericytes recruitment.	(151)
VEGF-A	Yes	Homozygous and heterozygous mice have defective vasculogenesis, decreased number of cells in the yolk sac blood islands, less vessel invasion in the forebrain, reduced number of ECs, and defective vascular patterning.	(129;153)
bFGF	No	Mice are normal with only decreased vascular tone and low blood pressure.	(154)
Ang1	Yes	Die during embryo development embryonic day (ED) 12.5 due to poor vascular remodeling and defects in EC adhesion and spreading.	(155)
Ang2	Yes	Lethal with eye, lymphatic, and kidney defects. Lymphatic defect can be rescued by Ang1 cDNA, but not the eye phenotype.	(156)
Semaphorin 3E	Yes	Same phenotype described for Plexin D1-KO mice.	(157)
ephrinB2	Yes	Mice die at mid-gestation from vascular anomalies and lack of remodeling.	(158;159)
Growth Factor receptors			
endoglin	Yes	Normal vasculogenesis but are embryonically lethal as a result of defective vascular remodeling and smooth muscle cell differentiation.	(152)
PDGFR-β	Yes	Embryonic lethality due to lack of pericytes recruitment which causes the formation of microvascular	(160)

		aneurysms.	
VEGFR-2	Yes	Haemangioblasts fail to differentiate into ECs.	(161;162)
VEGFR-1	Yes	Vascular lethal defect that is due to an increase in the number of haemangioblasts.	(163;164)
FGFR-1 or -2	Yes	Leads to embryonic lethality prior to gastrulation.	(165;166)
FGFR-3	No	Mice have skeletal abnormalities and deafness.	(167)
Tie2	Yes	Mice die between ED 9.5 and 12.5 due to lack of remodeling of the primary capillary plexus. The heart also appears to have defects with poor associations between ECs and underlying matrix.	(168;169)
Tie1	Yes	Mice die between ED13.5 and the immediate post-natal period due to severe hemorrhages and edema suggesting defective vessel integrity. Mice also have increased vessel density.	(168)
Neuropilin1	Yes	Deletion leads to abnormal vascular development and poor branching in the brain subventricular zone.	(170)
Plexin D1	Yes	Abundant branching in the intersomitic vessels with loss of normal vascular patterning.	(171)
EphB4	Yes	Mice die at mid-gestation from vascular anomalies and lack of remodeling.	(158;159)
Intracellular kinases			
PKG	No	Mice have decreased adult ischemia-induced angiogenesis.	(172)
ShcA	Yes	Lethal due to defect in Ras-MAPK pathway.	(173)

	Ras-GAP	Yes	Lethal due to defect in Ras-MAPK pathway.	(174)
	MEK kinase 3	Yes	Lethal due to defect in Ras-MAPK pathway.	(175)
	Src	No	Viable but have impaired VEGF-induced permeability and angiogenesis.	(176)
	Yes	No	Viable but have impaired VEGF-induced permeability and angiogenesis.	(176)
	Fyn	No	Mice are normal.	(176)
	Etk	No	Mice are normal but have impaired hind-limb ischemia-induced angiogenesis and arteriogenesis.	(177)
	PAK1	No	Mice are normal.	Unpublished
Matrix proteins				
	fibronectin	Yes	Mice die by ED 9 due to defects in somitogenesis and vascular development. Very similar to α5-KO mouse phenotype.	(178)
Integrins				
	α1	No	Viable and fertile and wound healing is not altered.	(179)
	α2	No	Viable and fertile, however pathological angiogenesis is altered (tumor growth and tumor-induced angiogenesis is reduced).	(180)
	α3	Yes	Lethal phenotype within hours after birth due to aberrant basement membrane organization. They also have dilated and reduced kidney glomeruli.	(181;182)
	α5	Yes	Mice die at ED10-11 because yolk sac and embryonic vascular network fails to properly form.	(183)

α6	Yes	Lethal phenotype within hours after birth due to aberrant basement membrane organization. Also have severe epidermal blistering.	(184)
α3 & α6	Yes	Double knockout has severe skin blistering but no known blood vessel defects.	(185)
αv	Yes	Developmental vasculogenesis and angiogenesis proceed normally until ED9.5. Between ED10.5 and ED11.5, 70% of embryos die due to a placental defects. By E12.5, the surviving embryos develop cerebral hemorrhage and die soon after birth.	(186)
β1	Yes	Mice die during the early post-implantation period prior to vascular development.	(187)
β3	No	Viable and fertile.	(188)
β4	No	Severe epidermal blistering defects but no defect in either developmental vasculogenesis or angiogenesis.	(189)
β5	No	Viable and fertile.	(190)
β3 & β5	No	Double knockout is viable and fertile and has normal developmental angiogenesis. Interestingly, mice demonstrate increased pathological angiogenesis.	(189;191)
β8	Yes	Very similar to αv. Mice die due to impaired blood vessel support.	(192)
Adhesion molecules			.
VE-cadherin	Yes	Lethal during early days of development. ECs form primitive vascular plexus but vascular remodeling is missing. ECs detach from one another causing vessels	(193)

		to regress and collapse.	
PECAM1	No	Viable and fertile. Also have altered coronary circulation.	(194)
ICAM1	No	Viable and demonstrates decreased growth factor-induced angiogenesis.	(195)
Transcription factors			
HIF1α	Yes	Mice die by ED11 due to extensive vascular defects and cardiovascular malformation.	(196)
HIF2α	Yes & No	Mixed results, but generally mice die *in utero* or shortly after birth due to bradycardia, respiratory distress, or improper vascular connections.	(197;198)
Other			
Retinoic Acid	Yes	Capillary plexus formation occurs, but capillary plexus remodeling and later stages of vessel assembly are disrupted.	(199)

Abbreviations: TIMP, tissue inhibitor of matrix metalloproteinase; MT-MMP, membrane associated matrix metalloproteinase; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; PKG, cGMP dependent protein kinase; Etk, epithelial tyrosine kinase; PAK1, p21 activated kinase.

protein fragments v) transcription factors, and vi) intracellular kinases and proteins. It is beyond the scope of this section to discuss all of these classes in detail; however these reviews provide an overview of these signaling components(15;37;127;128). Only a few key growth factors, integrins, MMPs, and intracellular kinases that regulate angiogenesis will be briefly discussed.

2.6.1 VEGF

VEGF-A (commonly referred to as VEGF) is a heparin-binding, EC-specific growth factor that has been extensively studied and demonstrated to have an essential role in development and proper patterning of new and immature vasculature(129-131). It was originally discovered as vascular permeability factor (VPF), for its ability to induce localized edema and increased permeability(132). Since all VEGF isoforms contain eight conserved cysteine residues they are also considered to be members of the PDGF family(12). The isoforms in this family include VEGF (VEGF-A through VEGF-E, which are highly homology), PDGF, and P1GF. VEGF isoforms are active as homodimers, but some evidence also suggests that they can be active as heterodimers. The isoform VEGF-A, and to a lesser extent VEGF-B and VEGF-C, have been widely implicated in vasculogenesis and the development of primitive vasculature(12). Expressed throughout the embryo except in ECs, VEGF specifies where the primordial dorsal aorta and other major vessels will form during embryogenesis. VEGF gene deletion *in vivo* induces a lethality that demonstrates the essential role of VEGF in vascular development(129). Examination of VEGF-KO mutant embryos reveals that normal vasculogenesis is severely impaired and vitelline veins do not coalesce with the *de*

novo circulation in the yolk sac (Table 2-3). The requirement for VEGF also appears to be dose-dependent since heterozygous mutant embryos also fail to induce vascular pattering and form yolk sac blood islands(153).

The biological effects of VEGFs are mediated via three specific cell surface receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1) and VEGFR-3 (Flt-4)(12). All three consist of an extracellular domain comprising seven Ig-like domains, a transmembrane domain, and a cytoplasmic kinase domain. While VEGFR-3 is primarily expressed in the lymphatic system, VEGFR-1 and VEGFR-2 are mainly expressed on ECs, although other cell types of hematopoietic origins can also express these receptors. Disruption of genes encoding VEGFR-1 and VEGFR-2 leads to disrupted vascular development and embryonal death(161;164). In the absence of VEGFR-2, haemangioblasts fail to differentiate into ECs. However, in the absence of VEGFR-1, the vascular defect is instead due to an increase in the number of EC progenitors, suggesting that the biological function of VEGFR-1 is to suppress vasculogenesis and sequester VEGF (Table 2-3).

VEGF ligand binding to VEGFRs induces receptor dimerization and autophosphorlyation of cytoplasmic catalytic tyrosine kinase domain. Various signaling molecules can interact with the newly phosphorylated receptor residues, which in turn leads to a cascade of signaling events (reviewed in (12;200)). Molecules such as focal adhesion kinase (FAK), Src family non-receptor tyrosine kinases, Shc-like protein (Sck), phospholipase C-γ (PLC-γ), the MAPK p38, and phosphoinositide kinase-3 (PI3K), are among those that are directly activated by phosphorylated VEGFR-2. As illustrated in Figure

2-2, many of these molecules have the capacity to activate other molecules in the cell, which subsequently induce a variety of unique cellular events. Among those reported including gene expression, altered cytoskeletal dynamics, cell proliferation, cell migration, and differentiation(12;21). Furthermore, VEGFR can also associate with integrins to potentate adhesion-induced angiogenic signaling. For instance, VEGF activation of VEGFR-2 can in turn activate Src and potentate αvβ5 integrin signaling. This cooperation in signaling not only induces angiogenesis *in vivo*, but also protects cells from the extrinsic apoptosis pathway, thus enhancing cell survival(201).

2.6.2 bFGF

Basic-FGF, also known as fibroblast growth factor-2, was the first pro-angiogenic molecule to be identified(11). At present, the FGF family consists of at least 20 factors that are nearly identical in their primary amino acid sequences. Surprisingly, the classical FGFs, acidic-FGF (aFGF) and bFGF, lack cytoplasmic sequences for extracellular export, in contrast to most other angiogenic growth factors(12). Furthermore, FGFs bind with high affinity to heparin sulfate proteoglycans (HSPGs) that are located on the surface of most cells and in the ECM. These bound FGFs are among a pool of other growth factors that can be released in a regulated manner by the action of heparanases and ECM proteases.

There are several similarities between VEGFs and FGFs. For example, like VEGFs, FGFs also mediate their biological effects by interacting with their four structurally related

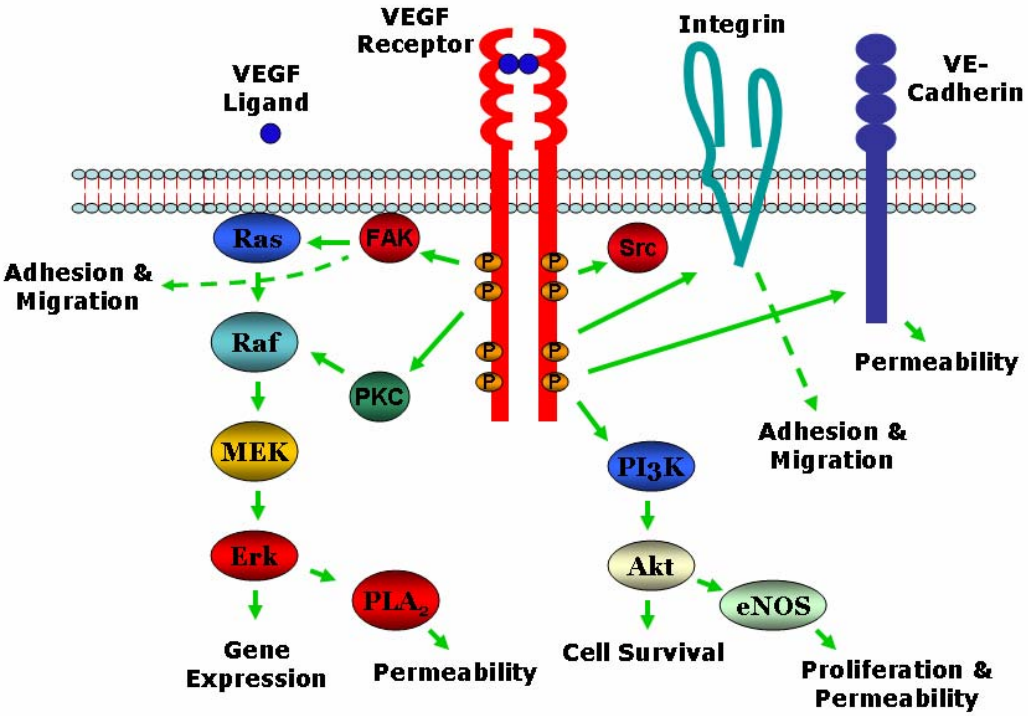

Figure 2-2. VEGF signaling in ECs. VEGF ligands facilitate VEGF receptor typrosine kinase dimmerization and autophosphorlyation. Phosphorylated receptors activate downstream signaling molecules such as FAK, PKC, PI3K, and Src. FAK is essential for forming EC focal adhesion complexes and facilitating adhesion, migration, and gene expression via Ras/Raf/MEK/ERK signaling. ERK activation can also induce prostacyclin production to induce vascular permeability. PI3K activation induces Akt survival pathways, and stimulates eNOS production to facilitate proliferation and permeability. Moreover, activated VEGF receptors can both directly and indirectly interact with integrins and cell adhesion molecules, such as VE-Cadherin, to further facilitate EC adhesion, migration, and permeability. PI3K, phosphoinositide-3 kinase; PLA_2, prostacyclin LA_2; eNOS, endothelial nitric oxide synthase.

receptor tyrosine kinases FGFR-1, -2, -3, and -4. These receptors and their splice variants are widely expressed and display different ligand interactions(202). Like VEGFRs, gene knockout studies have revealed that both FGFR-1 and FGFR-2 are essential for the development of the vasculature in the mouse embryo (Table 2-3)(165;166). Moreover, FGFs dimmers also can complex with their dimmerized FGFRs to induce receptor intermolecular autophosphorylation. These events in turn lead to activation of molecules like Src, Ras, p38, PLCγ, and PI3K (reviewed in (12;200)). Finally, both bFGF and VEGF can induce angiogenesis *in vivo* in the chicken chorioallantoic membrane, are secreted at high levels by many tumor cell lines, and are elevated in the serum of a large subset of cancer patients who have solid tumors(68;203;204).

However, FGFs also differ from VEGFs in various ways. For example, unlike the embryonic lethality induced by the loss of VEGF, genetic ablation of bFGF yields mice that are essentially normal, with only slightly reduced vascular tone and blood pressure(154). This suggests that there are significant redundancies among the FGF protein family that may compensate for the loss of bFGF during vasculogenesis and angiogenesis(12). Furthermore, bFGF and VEGF can both induce the Ras-MEK-MAPK signaling cascade, but in some instances through exclusive pathways. For instance, Hood et al. report that unlike VEGF which potentiates αvβ5-induced MAPK signaling through FAK, bFGF instead facilitates αvβ3-induced MAPK signaling through PAK1 activation(201). Similar studies also demonstrate that bFGF can mediate cell survival by protecting cells from the intrinsic apoptosis pathway, while VEGF protects cells against the extrinsic apoptosis pathway(205).

2.6.3 Angiopoietins

In addition to VEGFs and FGFs, angiopoietins comprise another important group of angiogenic growth factors that can also regulate EC signaling and angiogenesis. Ang1-4 are very similar in peptide sequence and protein structure and they can regulate angiogenic remodeling in a context-dependent manner(206). These growth factors induce their cellular effects through the Tie2 receptor, a receptor tyrosine kinase that is mainly expressed on ECs. Ang2 is expressed and stored in ECs, but its Tie2 mediated signals are contextual (sometimes it acts as an agonist and sometimes as an antagonist)(207;208). On the other hand, Ang1 is primarily expressed by non-endothelial mesenchymal cells, can be incorporated into the ECM, and is an agonist for Tie2 signaling(209). In addition to Tie2, Tie1 is another Tie receptor which can induce ligand-independent intracellular signals that mimic Tie2 signaling(206). However, it is disputed whether any of the angiopoietins can bind to Tie1(209;210). In both cases, signaling through the Tie receptors has been shown to regulate multiple steps in vessel development, maturation, and angiogenic remodeling.

The importance of angiopoietins and their receptors in vascular development, as well as in mediating cell-cell and cell-matrix interactions, is highlighted by the embryonic mouse lethal phenotypes induced in mice lacking either the Tie receptors or angiopoietin ligands (see Table 2-3). Although vasculogenesis in these mice proceeds normally, the essential angiogenic remodeling and maturation steps that follow are severely impaired(206). As a result, angiopoietin and Tie2 deficient mice present with severe hemorrhages, edema, poorly remodeled vasculature, and significant defects in EC adhesion and spreading. Interestingly,

Ang2-KO mice also demonstrate defects in their lymphatic vessels, as well as in their eyes and kidneys, which suggests that angiopoietin signaling is also likely to be important for other systems(156).

In addition to their critical functions during vascular development, angiopoietins and the Tie receptors also play an important role in EC signaling in the adult. Upon angiopoitein binding, the Tie-2 receptor dimerizes and its cytoplasmic tyrosine kinase domain undergoes autophosphorlyation (reviewed in (206)). Activated Tie2 can associate with other cell surface molecules (like integrin α5β1) as well as a number of cytoplasmic proteins, including Dok-R, GRB2, and PI3K, which in turn activate downstream signals(206;211). For example, Dok-R activates PAK1, GRB3 facilitates Ras-MAPK activation, and PI3K induces the activation of other intracellular kinases such as FAK, Akt, and ERK1/2. These multiples and diverse signaling pathways are responsible for facilitating angiopoietin-induced EC survival, migration, sprouting, tube formation, maturation, and remodeling. Furthermore, through the mechanisms are not fully understood, signaling through Tie2 can also up- or down-regulate gene expression. For example, angiopoitiens can induce vessel remodeling by increasing metalloprotease and plasmin expression(212;213), can mediate anti-inflammatory effects by inducing decreased ICAM-1, VCAM-1, and IL-8 expression(214;215), and can increase vessel permeability by inducing PECAM-1 and VE-cadherin expression(216).

2.6.4 Integrins

Integrins are a family of heterodimeric, transmembrane glycoproteins that are primarily

involved in cell-to-ECM adhesion and signaling(127). They are composed of a single α-subunit that is not-covalently associated with a single β-subunit. In mammals, there are 8 different types of β-subunits and 18 types of α-subunits(217). Of these subunits, only 6 α-subunits and 4 β-subunits are known to be expressed on ECs. These surface expressed subunits can assemble to make at least 8 different and unique integrins, including α1β1, α2β1, α3β1, α5β1, α6β1, α6β4, αvβ3, and αvβ5(127) (Figure 2-3). The heterodimer composition of each of these integrins confers a certain degree of ligand specificity, although some integrins bind similar substrates and others can bind many substrates(218). For example, α1β1 and α2β1 are both relatively specific integrins that predominantly bind to collagen, but can also bind to laminin. Similarly, α3β1, α6β1, and α6β4 predominantly bind to laminin, however α3β1 is less specific in its binding and can also bind to thrombospondin(127). Finally, α5β1, αvβ3, and αvβ5 can bind to ECM via an arginine-glycine-asparagine (RGD)-binding site (reviewed in (217;219)). Thus ECM matrix proteins, like fibronectin, that contain an RGD sequence, will bind to these integrins. However, of this group only α5β1 selectively binds fibronectin. Integrin αvβ5 instead binds a similar protein called vitronectin, and αvβ3, in addition to binding to both fibronectin and vitronectin, also binds to vWF, thrombospondin, osteopontin, and denatured collagen(127).

Moreover, results from gene knockout studies of integrin subunits that are expressed on ECs have been both compelling and complex. First, certain integrin subunits clearly have an essential role in development. Accordingly, genetic deletion of the α-subunits α3(181),

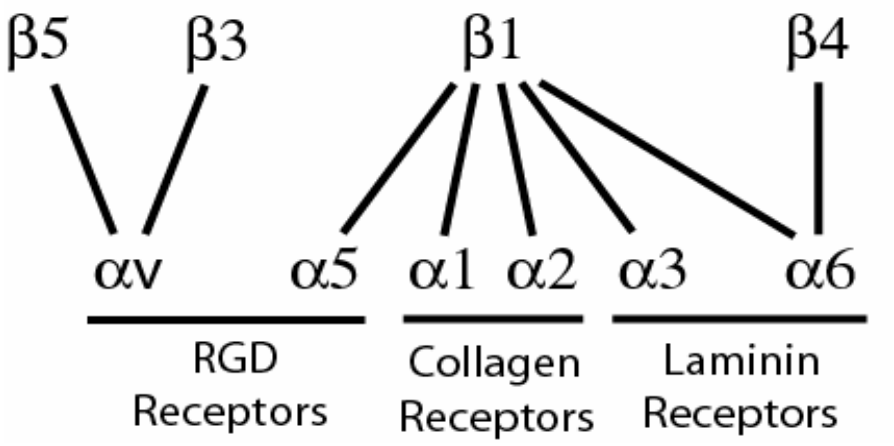

Figure 2-3. EC integrin subunit pairing and classification. ECs express different α and β integrin subuinits that can assemble into at least 8 different integrins. EC integrins can bind a wide range of ECM proteins. For example, αvβ3, αvβ3, and α5β1 are classified as RGD receptors because they bind ECM proteins such as fibronectin and vitronectin. Integrins α1β1 and α2β1 primarily bind collagen, but can also bind laminin, while α3β1, α6β1 and α4β6 primarily bind to laminin.

α5(183), α6(184), and αv(186), and the β-subunits β1(187) and β4(189), all yield lethal phenotypes in mouse embryos (Table 2-3). However, of these subunits, only α3, α5, αv, and β1-KO mouse embryos demonstrate impaired blood vessel development. Integrin α6 and β4-KO mice may also have impaired developmental angiogenesis, but so far the lethal phenotype is thought to be due to a distribution of the basement membrane integrity. Moreover, mice null for α1(179), α2(180), β3(188), and β5(190) are all viable and fertile, suggesting that these integrin chains are not essential for developmental angiogenesis. Interestingly, when pathological angiogenesis is invoked in these seemingly normal mice, a range of observations have been reported. In α2-KO mice, no difference is reported, and again these mice appear to be respond identical to their wildtype control(180). However, in α1-KO mice, pathological tumor growth and tumor-induced angiogenesis are dramatically reduced(220). On the other hand, pathological angiogenesis in β3- and β5-KO mice is augmented in response to ischemia and tumors(191). Thus, collectively it is clear from these gene knockout studies that the role of integrins in angiogenesis is complex. Furthermore, it is important to note that integrin subunits that are not essential for development, may still contribute to pathological forms of angiogenesis.

The complexity associated with integrins is also apparent in the molecular signals they transduce in ECs. Under normal conditions, integrins on ECs are generally thought to be in the 'off' state. In fact, expression of integrins αvβ3, αvβ5, and α2β1 is barely detectable in the quiescent endothelium, and is upregulated various fold upon EC sprouting (221). Integrin αvβ3 and α5β1 expression is also upregulated upon stimulation of ECs with growth factors and during angiogenesis(219). On the molecular level, activation of ECs

induces changes in integrin conformation, which in turn allows it to bind ECM. This phenomenon is often referred to as integrin inside-out signaling(218). As reviewed in (127;218), upon binding to ECM, integrins can cluster and induce a variety of cellular events, including the recruitment and activation of various important cytoplamsic signaling molecules (i.e. FAK, Rho family GTPases, Src). As illustrated in Figure 2-4, these molecules in turn transduce intracellular signaling cascades that involve the activation of cytoplasmic kinases such as PAK1 and components of the MAPK signaling cascade. Additionally, integrins can also stimulate lipid metabolism (e.g. phosphatidylinositol-4,5-biphosphate (PIP2) synthesis), induce ion transfer (e.g. Na^{2+}/H^{+} transfer, and Ca^{2+} influx), change intracellular pH, and alter EC morphology through interactions with the actin cytoskeleton via proteins such as paxillin, vinculin, and talin (except for integrin α6β4 which instead affects EC morphology by linking to intermediate filaments)(127;218;222). All of these alterations have repeatedly been demonstrated to be essential for proper EC adhesion, migration, growth and differentiation.

A large body of evidence also indicates that integrins not only signal on their own but also facilitate growth factor receptor signaling(223;224). For example, Hood et al. demonstrate that αvβ3 preferentially mediates bFGF signaling through FAK and PAK1 activation, while αvβ5 preferentially mediates VEGF signaling through FAK activation(201). Similarly, α5β1 is necessary for optimal activation of Tie2 by its ligand Ang1(225). The selectivity in integrin/receptor tyrosine kinase associations may be a result of direct binding. For example, the α5β1 integrin co-immunoprecipitates with Tie2, while αvβ3 integrin

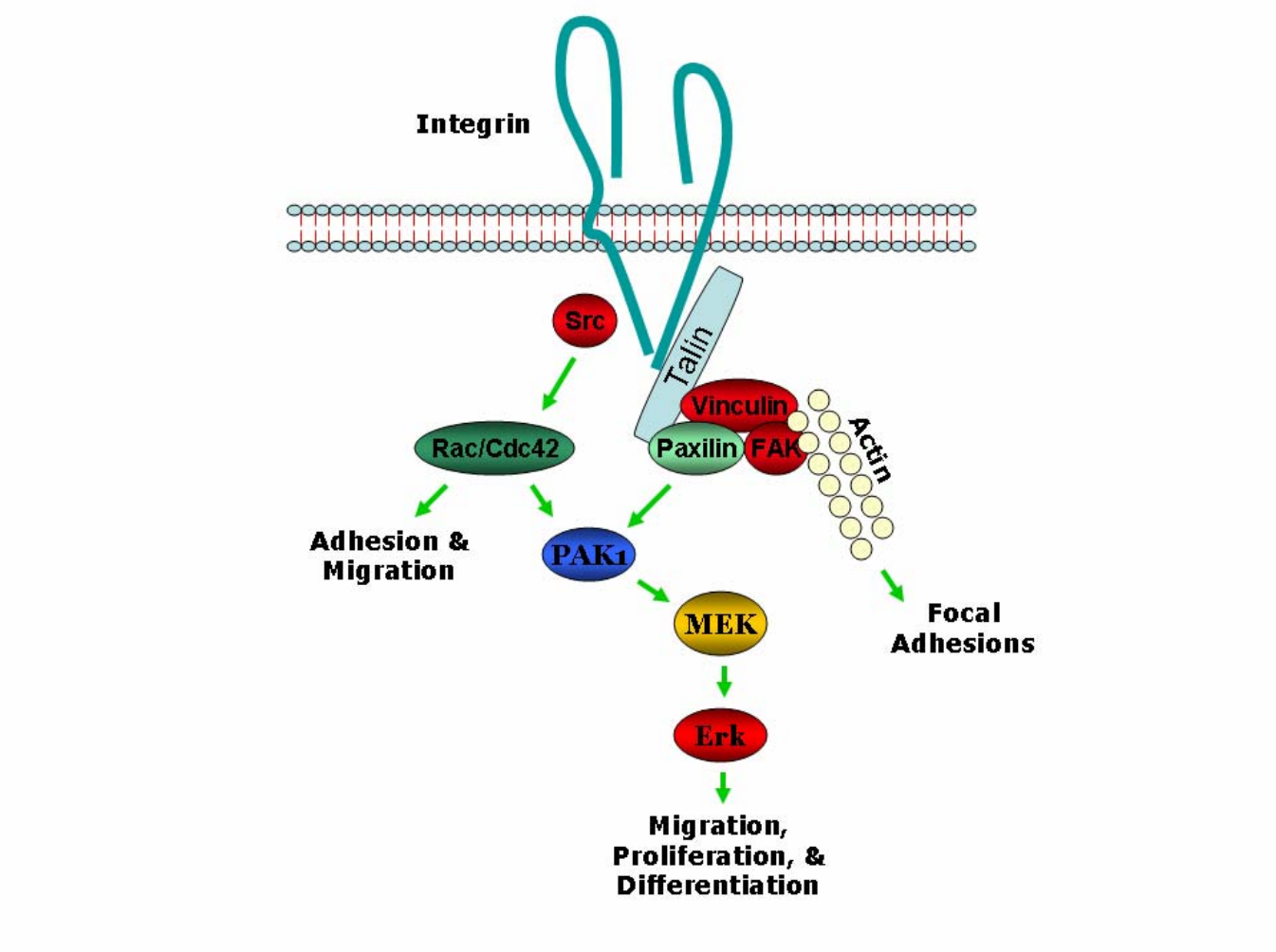

Figure 2-4. Integrin-mediated EC signal transduction. Upon ECM binding, EC integrins can activate several intracellular kinases. For example, integrins can directly activate Src, which can in turn activate Rho GTPases such as Rac/Cdc42. Alternatively, integrins can stimulate the formation of focal adhesion complexes that are composed of talin, vinculin, paxilin, FAK, and actin. Downstream to these events PAK1 is activated and induce various signaling pathways, including MAPK signaling. Collectively, integrin signaling induces various EC functions, including adhesion, migration, proliferation, and differentiation.

associates with the PDGF receptor(225;226). In either of these cases, the integrin interaction appears to be essential in mediating growth factor-induced EC signaling and angiogenesis.

2.6.5 MMPs

MMPs are zinc-endopeptidases that are key modulators of numerous physiological and pathological processes. Under normal conditions, MMPs modulate skeletal bone formation, coagulation, cellular migration, inflammation, wound healing, reproduction, growth and development, and physiological angiogenesis(227). Pathologically, MMPs also play an important role in various lung diseases, atherosclerosis, aneurism, sequelae of myocardial infarction, inflammation, infective diseases, degenerative diseases of the brain, tumorgenesis, and cancer metastasis(227;228). In humans, these effects are regulated by 24 known members of the MMP family. To induce their effects, these MMPs bind to an array of substrates, including ECM proteins, growth factors, soluble proteins, and cell surface receptors(228).

Expression of most MMPs is tightly regulated at the transcriptional level by growth factors, hormones, cytokines, cell-to-cell and cell-to-matrix interactions(229). The majority of MMPs are secreted as zymogens in propeptide form that require activation in order to cleave their substrates. Extracellular activation of MMPs is complex, and involves chemical or proteolytic cleavage of the prodomain of proMMP zymogens, and most MMPs are mutually activated by other MMPs(227). For example, MMP9 can be activated by other MMPs such as MMP2, MMP3, and MMP7(228). In some cases, pericellular activation of

proMMPs may also depend upon membrane-associated MMPs (MT-MMPs). For instance, both proMMP2 and proMMP13 are both activated by MT1-MMP (MMP14)(228). ProMMP2 activation also requires the presence of TIMP2, which forms a complex with proMMP2 and MT1-MMP, to yield the active MMP2 zymogen(230). In addition, TIMPs (TIMP1-4) are also responsible for the inhibition of activated MMPs, and affect biological functions such as cell proliferation, apoptosis, and angiogenesis(228).

MMPs are subdivided into different categories. The most common classification is based on historical assessments of substrate specificity and cellular localization of the MMPs. In this classification there are 5 main categories: i) collagenases, ii) gelatinases, iii) stromelysins, iv) matrilysins, and v) membrane bound MMPs (MT-MMPs). For brevity this introduction will only overview gelatinases, however for a more in-depth review of all the MMP sub-categories see(227).

There are two types of gelatinases. MMP2 (gelatinase A) is a non-glycosylated 72-kDa monomer enzyme that mediates most of its functions through the proteolygic degradation of ECM proteins such as elastin, fibronectin, laminin, and collagens IV, V, VII, and X(227). It is abundantly expressed by normal ECs, fibroblasts, and epithelial cells, as well as many transformed cells(231;232). *In vivo*, MMP2 has been detected at sites of tumor growth, aneurysm formation, atherosclerosis, and myocardial infarctions(233;234). It is hypothesized that increased MMP2 at these sites increases ECM rapid turn-over, remodeling, and the accumulation of phagocytic macrophages. Pharmacological inhibition and gene deletion of MMP2 can protect against these effects, reduces the incidence of lethal cardiac

rupture following myocardial infarctions, and protects against experimentally-induced abdominal aortic aneurysms. MMP2-KO mice also have reduced tumor onset and growth, and tumor-induced angiogenesis(134). However, genetic deletion of MMP2 in mice leads to very few developmental side-effects, and mice are viable and fertile (Table 2-3). The lack of a developmental phenotype suggests that other MMPs, such as MMP9 (gelatinase B), can compensate for the loss of MMP2 during development.

MMP9 is the other member of the MMP gelatinase family. It is a glycosylated 92-kDa enzyme that exists in both monomer and dimer forms. Like MMP2, it also mediates its effects by degrading ECM proteins such as collagen types IV, V, and XI, aggrecan, and elastin(227). However, unlike MMP2 it is not expressed by ECs, and instead it is mainly expressed by normal leukocytes and various types of transformed cells(235). MMP9 can also activate other molecules such as cytokines IL-1β and TGF-β, and convert plasminogen to angiostatin (a potent *in vivo* angiogenesis inhibitor)(220;227). Moreover, like MMP2-KO mice, MMP9-KO mice are also viable, but exhibit several developmental abnormalities (Table 2-3). For example during development, MMP9-KO mice have delayed and abnormal vascular and ossification patterning in the skeletal growth plate. In adult mice this defect leads to moderate skeletal abnormalities due to delayed growth in the cartilage ossification hypertrophic zones(135). In pathological states, as in MMP2-KO mice, loss of MMP9 alters ECM remodeling after myocardial infarctions and reduces the severity of atherosclerotic plaques(136;236).

Despite their largely overlapping functions, MMP2 and MMP9 may be differentially regulated, and in some cases can facilitate opposing functions. For example, compared to MMP2, MMP9 is reported to be more highly upregulated upon cellular stimulation with molecules such as epidermal growth factor (EGF), PDGF, bFGF, VEGF, IL-1α and β, and TNF-α(228). Moreover, unlike MMP2 pro-angiogenic and tumorgenic effects, MMP9 mediates both mediate pro- and anti-tumorgenic effects. Furthermore, MMP2 promotes platelet aggregation, while MMP9 does the opposite by inhibiting platelet aggregation(228).

2.6.6 PAK1

The serine/threonine kinase PAK1 belongs to a family that consists of six known members, PAK1 through PAK6(237;238). Each member of this family is encoded by a distinct gene and the differences in their N-terminal regions contribute to their ability to recruit different intracellular molecules and activate distinct signaling pathways(239). PAK1 activation is regulated by small GTP-bound GTPases(240). The Rho family GTPases Cdc42 and Rac can cause autophosphorlyation of PAK1, and are stimulated by multiple growth factors (such as EGF, PDGF, HGF, VEGF, bFGF, and heregulin), as well as integrins and G protein coupled receptors(201;241-243). Alternatively, PAK1 can be activated by adapter molecules (such as Nck and the guanine nucleotide factor Pix), AGC protein kinase family members (such as Akt-1, PDK1 and cAMP-dependent protein kinase), lipids (such as phosphorylated 3-phosphoinositid-dependent kinase 1), and sphingosine (as well as related long-chain sphingoid bases; reviewed in (239)). As illustrated in Figure 2-5, activated PAK1 also has numerous downstream substrates that affect a diverse set of cellular functions

(including cellular growth, proliferation, and actin cytoskeleton reorganization), thus making it apparent that PAK1 is likely an important signaling molecule. For an in depth overview of PAK1 functions, see (237;239).

Evidence to support the role of PAK1 in cellular growth and proliferation is demonstrated in its involvement in tumor cell proliferation and cancer progression(239). For instance, Ras-mediated signaling, which induces cellular transformation and proliferation, also induces PAK1 activation and MAPK signaling(244). The expression of catalytically active PAK1 in tumor cells can on its own also induce MAPK signaling and stimulate anchorage-independent growth(245). PAK1 can also enhance cell survival by inactivating the pro-apototic functions of BCL2 family member proteins(246).

In addition to these functions, PAK1 also plays a significant role in cellular migration and actin cytoskeleton reorganization(239). It is known to facilitate these processes through multiple different mechanisms. For example, PAK1 can stimulate migration through activation of ERK1/2 and p38(247-249). The pro-migratory role of PAK1 can be inhibited in breast cancer cells by overexpression of a kinase-dead PAK1 mutant. On the other hand, PAK1 can also inhibit migration. This is mediated by PAK1 induced LIM kinase 1 activation. Activated LIM kinase 1 in turn phosphorylates and inactivates cofilin and induces actin filament depolymerization(250;251). Therefore, this demonstrates that the effects of PAK1 on cellular migration depend on the context of cellular activation(252).

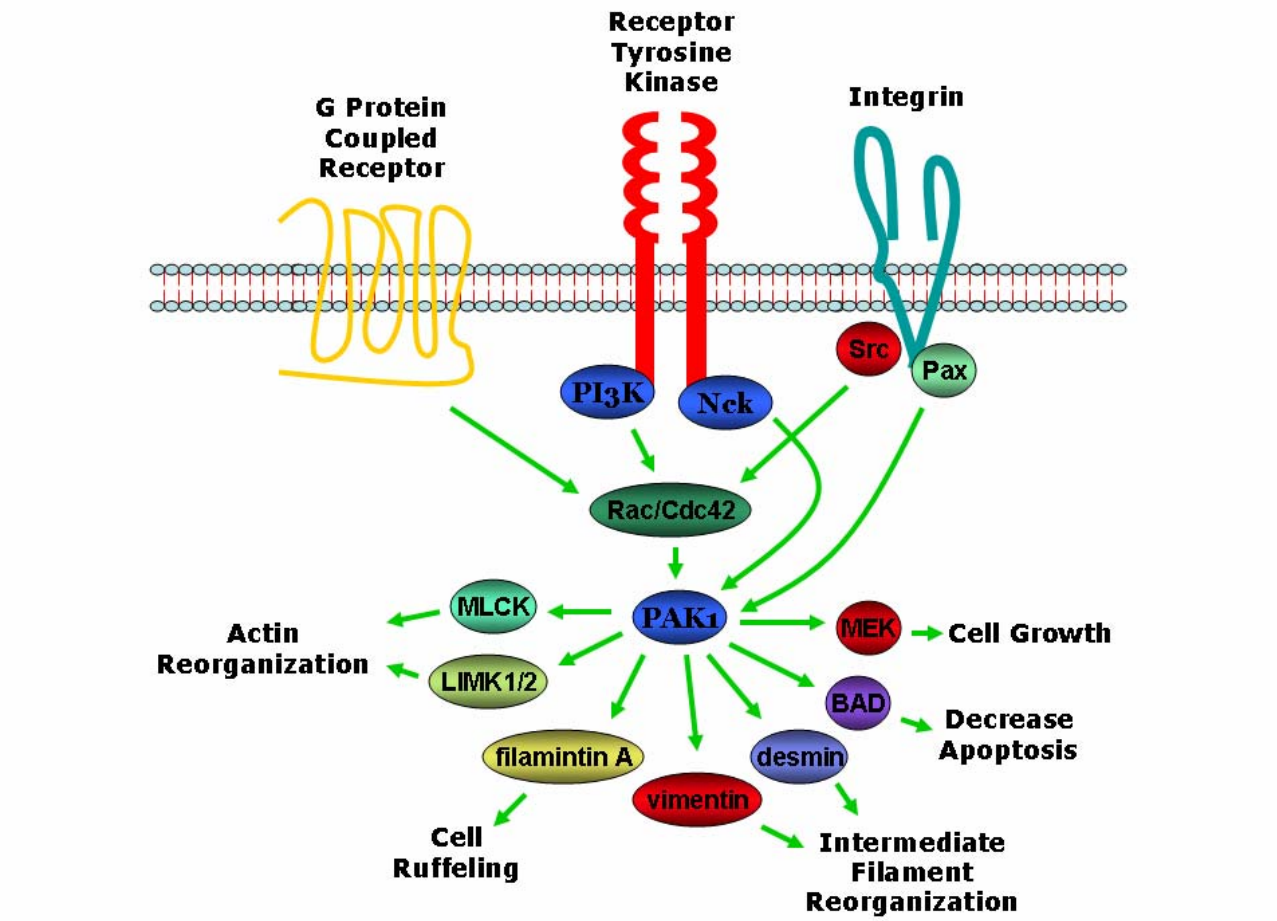

Figure 2-5. PAK1 is an important signaling molecule that affects various downstream molecules. G protein coupled receptors, receptor tyrosine kinases, and integrins can activate PAK1. In turn, PAK1 can phosphorylate and affect the function of various downstream molecules.

Accumulating evidence also suggests that PAKs plays an important role in regulating EC signaling and angiogenesis. Kiosses et al. provided the first evidence for this by demonstrating that constitutively activated and dominant negative forms of PAK both inhibit EC migration, therefore suggesting that proper PAK activation and signaling was essential for this process in ECs(250). Subsequently, dominant negative PAK was shown to strongly inhibit the interaction between Nck and PAK, and caused a significant decrease in growth factor-induced EC migration, contractility, and three-dimensional capillary structure formation(253). In this study, the disruption of the Nck/PAK interaction was also shown to decrease angiogenesis *in vivo* in a chick chorioallantoic membrane assay.

The role of PAK1 in angiogenesis was also confirmed by Hood el al., as well as others, who demonstrated that PAK1 acts as a point of convergence between growth factor-induced and integrin-induced angiogenic signaling(201). In these studies, PAK1 was shown to mediate VEGF-induced actin cytoskeleton reorganization, and bFGF-induced EC anti-apoptotic signaling. PAK1 mediates these effects by preferentially acting on the Ras-Raf-ERK1/2 MAPK signaling pathway, and by phosphorylating specific serine residues on either Raf1 or MEK1. Bagheri-Yarmand et al. also showed that PAK1 can participate in angiogenic growth factor expression, and mediated heregulin-induced VEGF expression(242).

2.7 Angiogenesis as a promising medicine

It is predicted that more than 500 million patients can benefit from anti- or pro-angiogneic therapy in the coming decades (254). However, proper *in vivo* delivery of therapeutics still remains an obstacle in the treatment of angiogenesis-contributing or angiogenesis-dependent diseases(255). Since the vascular endothelium in these diseases is the central target for intervention, much effort has recently been invested in determining how to best target this tissue in a safe, efficient, and selective manner.

2.7.1 Pro-angiogenic Therapy

Myocardial ischemia and peripheral vascular disease are two major causes of morbidity and mortality in humans(86). Over the past decade, advances in our understanding of neovascularizaiton have encouraged efforts to develop clinical interventions that may promote adaptive forms of angiogenesis in such cases of vascular insufficiency, wound healing, and tissue recovery(256). Two main strategies have been proposed to accomplish this: i) angiogenic gene therapy using viral vectors, or ii) administration of growth factors such as VEGF and bFGF at sites of ischemia(257). Preclinical animal models and preliminary clinical trials highlight the therapeutic promise associated with both of these strategies. For example, in a phase I clinical trial bFGF was slowly administered over a 4-6 week period in patients with ischemic heart disease and was found to significantly increase freedom from angina recurrence, increase myocardial neovasularization, and reduce myocardial ischemic area(258). In dogs, pre-existing collaterals demonstrated improved blood flow following administration of bFGF at sites distal from experimentally induced coronary artery constrictions(259). Single bolus administration of VEGF near the femoral

artery also alleviated experimentally induced hind-limb ischemia in mice, which models human peripheral vascular disease(260). Similarly, delivery of adenovirus carrying the VEGF gene to the diseased coronary arteries of heart disease patients decreases the incidence of angina (cited in (260)).

However, in order for angiogenic therapy to be more feasible and become a reliable method of therapy, clinical trials must demonstrate little or few acute side effects, enhanced specificity to targeted ischemic tissue so that unwanted angiogenesis is not stimulated, and sustained clinical benefit(256). Unfortunately, so far clinical trials have demonstrated a significant lack of treatment specificity, suboptimal delivery, and treatment-associated acute side-effects(261;262). Furthermore, delivery of VEGF and bFGF to ischemic tissue stimulates the growth of vessels that are morphologically and functionally less stable compaired to normal mature vessels(263). It is not yet clear whether increased systemic levels of angiogenic growth factors can induce decreased adhesion molecule expression in the systemic circulation, trigger dormant tumor growth, and accelerate vessel atherosclerosis(257;264). Current research is focusing on improving methods of treatment administration as well as optimizing viral gene transfer methods through modification of viral capsid proteins that enhance treatment selectivity and efficiency, and decrease host immunogenecity(265).

2.7.2 Anti-angiogenesis Therapy

Based on exciting preclinical data that surfaced in the 1990s(266), anti-angiogenic

therapy, which can alleviate aberrant forms of angiogenesis, has since been under development. Anti-angiogenic targets have been developed to interfere with angiogenic ligands like VEGF and PDGF, their receptors, or subsequent downstream signaling in ECs(267). As reviewed in (268), anti-angiogenic therapies have also been developed to upregulate or deliver endogenous angiogenesis inhibitors, inhibit EC integrins, neutralize matrix degrading enzymes, or inhibit EC-specific functions (such as proliferation, adhesion, migration, protease secretion, and differentiation). Several of these medicines are currently in clinical trials, and a few have already been approved for combination therapy with conventional treatments. See Table 2-4 for a list of agents currently being tested in clinical trials (http://cancertrials.nci.nih.gov/).

Anti-angiogenic therapy is currently in use to treat different types of angiogenesis-associated clinical disorders. For example, for the treatment of rheumatoid arthritis, anti-TNFα antibody infliximab (Remicade, Johnson & Johnson) not only neutralizes the effects of TNFα in inflammation, but also decreases angiogenesis and significantly reduces VEGF serum levels(269). Anti-angiogenic effects of infliximab are also noticed in other forms of arthritis, such as psoriatic arthritis(270). Similarly, becaplermin (Regranex, recombinant PDGF-β, Ortho-McNeil Pharmaceuticals) is FDA-approved to treat dermatological conditions such as diabetic neuropathic foot ulcers(271). For retinal diseases like AMD, intravitreal administration of anti-VEGF therapy (using pegaptanib sodium and ranibizumab) showed in phase III clinical trials to reduce the progression and severity of this disease(272).

Table 2-4. A partial list of anti-angiogenic agents undergoing clinical trial testing.

Category of Therapy	Drug	Description	Clinical Trial Phase
Blocks matrix breakdown			
	Dalteparin	Low molecular weight heparin	II
Blocks ECs directly			
	ABT-510	Small peptide thrombospondin-1 mimetic	I & II
	Lenalidomide	Thalidomide derivative	I, II, III, & IV
	Combretastatin A4 phosphate	Binds to tubulin and inhibits microtubule depolymerization	I & II
Blocks activators of angiogenesis			
	ADH-1 (Exherin™)	Competitively binds and inhibits cadherins	I
	AG-013736	Inhibits tyrosine kinase receptors	I & II
	Anti-VEGF Antibody (Bevacizumab; Avastin™)	Binds and inhibits action of VEGF	II, III, & IV
	BMS-582664	Inhibits VEGFR-2 activation	I & II
Inhibits EC-specific integrin/survival signaling			
	ATN-161	α5β1 integrin antagonist	I & II
	EMD 121974 (Cilengitide™)	Inhibits αvβ3 and αvβ5	I & II
Undefined method of action			
	Celecoxib, (Celebrex®)	Inhibits Cyclooxygenase-2	I, II, & III

Anti-angiogenic therapy has also been tested for its potential role in inhibiting tumor-induced angiogenesis and tumor growth. The goal of anti-angiogenic tumor therapy is to first effectively normalize tumor vasculature and then inhibit it all together(273). If this can be achieved it is hypothesized that tumors would be starved from essential nutrients and oxygen, stunt tumor growth, and eventually reduce tumor progression(113). The endothelium is a prime target for such therapy since unlike most tumor cells it is composed of cells that are diploid and genetically stable, thus making it less likely that they will develop resistance to therapy(68). Second, the tumor endothelium is pathologically activated unlike the normal endothelium, which is normally quiescent(274). Activated ECs are known to overexpress surface markers (such as integrin αvβ3, E-selectin, and Tie receptors) that are expressed to a lesser degree on the quiescent endothelium(68). Such differences in surface marker expression provide opportunities for selective targeting of the tumor endothelium in order to decrease therapy associated side-effects. Third, ECs are easily accessible to therapeutic agents through the blood stream(274). This is obviously beneficial since it allows for more efficient drug delivery at lower effective doses(13). Thus collectively, anti-angiogenic cancer therapy demonstrates enormous promise over conventional chemotherapy since it may overcome current problems of resistance, selectivity, and drug delivery(268).

The recent success of the FDA-approved humanized anti-VEGF monoclonal antibody bevacizumab (Avastin, Genentech, Inc.) has made the option of anti-angiogenic therapy even more attractive. In 2004 the FDA approved this drug for combination therapy of advanced colorectal cancer when in Phase III clinical trials it was found to significantly prolong

survival, and had a better ability to shrink tumors than standard therapy (5-fluorouracil, leucovorin, and oxaliplatin or ininotecan)(275). Bevacizumab also improves outcomes for patients with metastatic renal, ovarian, lung, and breast cancer(268). Furthermore, bevacizumab and its derivatives improve the condition of patients that have non-oncologic disorders. For example, in June, 2006, the FDA approved Ranibizumab (Lucentis, Genentech, Inc.) which is a Fab fragment derived from bevacizumab, for the use in AMD(276). Although patients that receive this therapy demonstrate significantly improved outcomes, the therapy so far has been prohibitively expensive(277).

Despite the success of these as well as other anti-angiogenic agents, clinical trials of these agents have also raised various important questions. For example, so far bevacizumab demonstrates an overall survival benefit in patients receiving combination therapy(268). It is still unclear why monotherapy with these agents has been less effective in human cancers, than in rodents(278). Second, bevacizumab causes some serious side effects, including increased incidence of elevated blood pressure, gastrointestinal bleeding, as well as intestinal perforation(279). This suggests that at least anti-VEGF therapy is not as EC-specific as previously thought. Third, clinical evidence suggests that targeting ECs in combination with other cell types, like stromal and hematopoietic cells, is more effective than therapies that target only ECs(267). This argues that despite the genomic stability of ECs, resistance may still occur. Finally, despite advances in anti-angiogenic therapy, cancer patients receiving angiogenesis inhibitors, even in combination with chemotherapy, still die(268). Therefore, although significant advances in anti-angiogenic therapy have been made in the past decade, their full potential in cancer therapy still remains to be determined.

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CHAPTER 3

Role of CIB1 in Vascular Biology

3.1 Identification of CIB1

Proper integrin function and signaling is critical for various physiological processes(1). Among these processes, integrin-mediated platelet aggregation is essential for efficient wound healing and vascular hemostasis(2). However, equally as important are pathological conditions that arise from disregulated platelet aggregation (e.g. hemorrhage, stroke, distal vein thrombosis, etc.)(3). Regulation of both physiological and pathological platelet functions are facilitated by the platelet fibrinogen receptor, integrin αIIbβ3(4). This integrin is specifically expressed in the megakaryocytic lineage, and its mechanism of regulation has been a subject of intense interest.

In an effort to understand how platelets regulate the function of αIIbβ3, Naik et al screened a human fetal liver cDNA library in a yeast two-hybrid system by using the αIIb cytoplasmic domain as bait(5). This screen yielded one positive clone; contained 855-base pairs that translated into a novel 191-amino acid, 22 kDa protein. This protein was identified as CIB (for calcium and integrin-binding protein), but is now commonly referred to as CIB1. Previous names for CIB1 also included CIBP, calmyrin, Kip, and ISBP(3). In subsequent biochemical studies, isothermal titration calorimetry (ITC) confirmed a high affinity association (0.7 μM) between CIB1 and αIIb(6). The specific residues in αIIb that are essential for CIB1 binding were also identified, thus further confirming the CIB1/αIIb interaction(7).

Early studies also demonstrated that CIB1 is N-terminally myristolated(8). This myrisotyl group appears to target CIB1 to the plasma membrane, since in lysates of nucleated (megakaryocytes and HeLa) and non-nucleated (platelets and erythrocytes) cells CIB1 almost entirely fractionates to the membrane(6). Moreover, disruption of the myristolation site on CIB1 by the addition of an N-terminal myc tag induces CIB1 cytoplasmic and nuclear localization(8). However, the effects of myristolation on CIB1 cellular localization are still not entirely clear, and it is unknown to what extent myristolation regulates the binding affinity of CIB1 to αIIb and other proteins.

Furthermore, upon identification of CIB1, sequence homology studies indicated that CIB1 belongs to a family of Ca^{2+}-binding proteins(5). Accordingly, CIB1 is homologous to calcineurin B (58% similarity) and calmodulin (56% similarity), both of which are known to regulate a variety of cytoplasmic proteins. Further, CIB1 sequence analysis showed that CIB1 contains 4 EF hand motifs, which may bind Ca^{2+}. Using a $^{45}Ca^{2+}$ blot overlay assay, recombinant CIB1 did bind Ca^{2+} like its homolog calmodulin(9). Subsequently, NMR studies reveled that CIB1 contains two non-Ca^{2+} binding EF hand motifs in addition to the two different EF hand motifs that bind Ca^{2+} in a regulatory manner(10). The dissociation constants for Ca^{2+} binding to these motifs are 2μm and 0.5μm(10).

3.2 Structure of CIB1

Gentry et al. describe the crystal structure of CIB1, which confirms that CIB1 contains four EF-hand motifs (EF1-4), two of which can bind Ca^{2+} (EF3 and EF4)(11). The

crystal structure (illustrated in Figure 3-1) also confirms that CIB1 is significantly homologous to various mammalian and non-mammalian EF-hand containing proteins. Interestingly, sedimentation equilibrium and gel filtration studies demonstrate that CIB1 exists as a monomeric protein. This is in contrast to Blamely et al., who later reported that CIB1 forms a head-to-tail dimmer(12). Nevertheless, CIB1 contains a hydrophobic channel on its surface that is preserved in other closely related EF-hand-containing proteins (e.g. calcineurin B and the neuronal calcium sensor (NCS) protein KChIP1), which may facilitate its ability to associate with various binding partners(11).

3.3 CIB1 binding partners

Since its discovery in platelets, CIB1 was found to be expressed in various human and murine cell types and tissue(6;13). The near ubiquitous expression of CIB1 also appears in several cancer cell lines (Bin Zhao and Cassandra Moran, unpublished data). The wide distribution of CIB1 expression prompted speculation that, like calmodulin and other EF-hand containing proteins, CIB1 binds and regulates the function of various intracellular proteins. In the past decade, nearly a dozen CIB1 binding partners have been identified (recently reviewed in (3)). It is beyond the scope of this section to discuss all of the CIB1-binding partners, but we will explore a subset of these proteins to highlight their diverse roles and different cellular localizations.

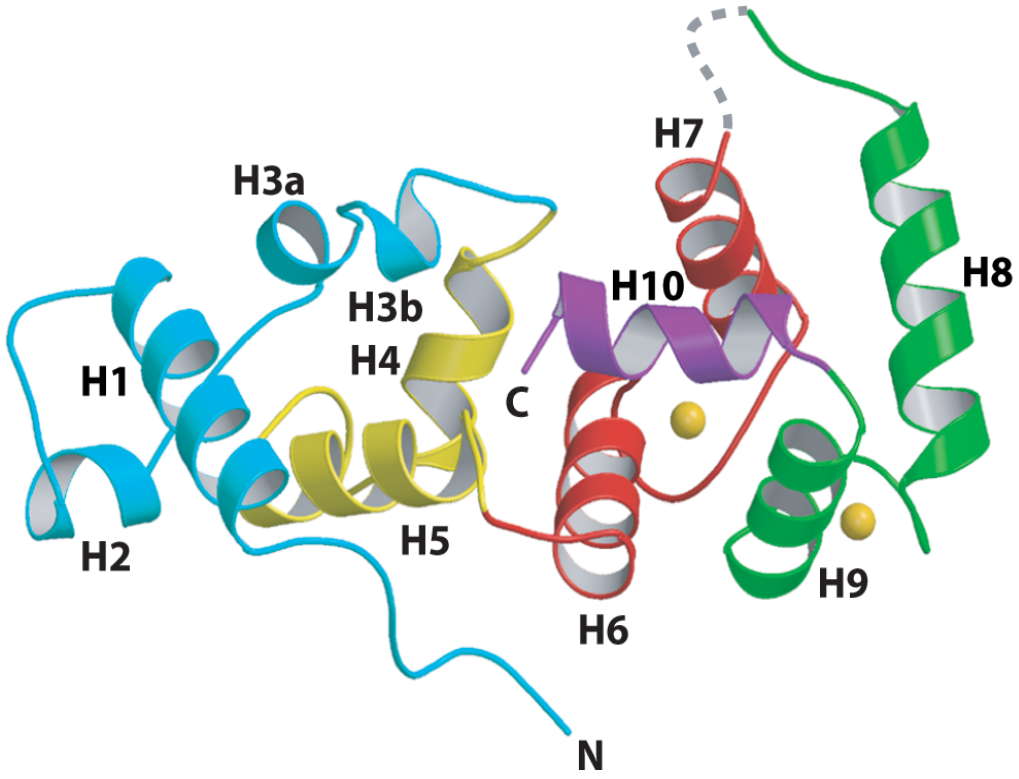

Figure 3-1. Ribbon diagram of CIB1 crystal structure. CIB1 contains four EF hand motifs, two of which bind Ca^{2+} (gold spheres). The N-terminal region and EF1 is blue, EF2 is yellow, EF3 is red, EF4 is green, and the C-terminus is purple. **This figure is published in the following reference: Gentry, H.R., Singer, A.U., Betts, L., Yang, C., Ferrara, J.D., Sondek, J., and Parise, L.V. "Structural and Biochemical Characterization of CIB1 Delineates a New Family of EF-hand-containing Proteins." *JBC*, 2005. 280: 9, 8407-8415. It is reprinted with permission from the American Society for Biochemistry and Molecular Biology Journals.**

3.3.1 PAX3 and DNA-PK$_{CS}$

Immunoflorescent microscopy of CIB1-transfected cells demonstrates that CIB1 can localize to the nucleus(8). Accordingly, CIB1 has been reported to bind and regulate the activity of several nuclear proteins that have diverse cellular functions. For example, CIB1 can bind PAX3, which is a homeodomain family transcription factor(14). Binding of CIB1 to PAX3 inhibits human and mouse primary myoblast differentiation. Similarly, CIB1 was also shown to associate with DNA-dependent protein kinase (DNA-PK$_{CS}$) in a yeast two-hybrid assay only. DNA-PK$_{CS}$ is a nuclear kinase that is important for the repair of double-strand breaks(15). Since the autophosphorylation of DNA-PK$_{CS}$ results in its inactivation(16), it is hypothesized that CIB1 binding to DNA-PK$_{CS}$ inhibits autophosphorylation and instead induces its activation(15).

3.3.2 InsP$_3$R isoforms

Isoforms of the inositol 1,4,5-triphosphate receptor (InsP$_3$R) that are localized to the endoplasmic reticulum (ER) are the primary cytosolic target responsible for the initiation of intracellular Ca^{2+} signaling (reviewed in (17)). This process is important since it can affect apoptosis, intracellular pH, exocytosis, and gene expression. To fulfill its effects, InsP$_3$Rs interact with several ER and cytosolic proteins. Recently, CIB1 was reported to bind all mammalian InsP$_3$R isoforms in a Ca^{2+}-dependent manner(18). In the absence of InsP$_3$ ligand, CIB1 was found to activate InsP$_3$Rs *in vitro*. However, in CIB1-transfected COS-7 and PC12 cells, CIB1 decreased InsP$_3$R activation. Thus these findings demonstrate that

CIB1 is an $InsP_3R$ protein ligand that can both stimulate and inhibit $InsP_3R$-mediated Ca^{2+} signaling.

3.3.3 Presenilin-2

A few reports have also implicated CIB1 binding to presenilin-2 (PS2) in a Ca^{2+}-independent manner(8;19-21). PS2 is an ER membrane protein that is mutated in early onset Alzheimer's disease (AD), which is a neurodegernative disorder characterized by dementia (reviewed in (22)). In a yeast two-hybrid screen, CIB1 bound to a cytoplasmic loop that is specific to PS2, and in cells this interaction enhanced cellular apoptosis(8). Immunohistochemistry of CIB1 showed that it is in part associated with diffuse and senile plaques in the forebrains of deceased human AD patients(20).

3.3.4 PAK1

CIB1 also binds and regulates the activity of various types of kinases(3). For example, CIB1 can inactivate kinases such as serum-inducible kinase (Snk or polo-like kinase 2)(23). On the other hand, CIB1 can also bind and activate the AGC protein kinase family member PDK1 (Bin Zhao, unpublished data). Most notably however, Leisner et al. provided the first evidence to show that CIB1 is an essential regulator of PAK1 activity in several different cell types including REF52 fibroblasts(24). In this study, CIB1 is found to specifically bind to the PAK1 NH2-terminus in a calcium-dependent manner at two putative binding sites (site I and site II). Interestingly, site I was found to be in close proximity to the

NH2-terminal p21-binding domain (PBD; where Rac/Cdc42 normally bind PAK1). Site II was found to be within the PAK1 NH2-terminal autoinhibitory domain. Since the crystal structure of PAK1 suggests that site I is more accessible than site II for binding(25), it is hypothesized that CIB1 binds to PAK1 in two-stages: i) first by binding the more accessible site I to induce a conformational change in PAK1, ii) then binding to site II where it may regulate PAK1 activation.

Using a series of *in vitro* kinase assays, Lesiner et al. demonstrate that CIB1 indeed regulates PAK1 activation levels and can inhibit Cdc42 binding to PAK1(24). This study also showed that in a Rac/Cdc42-independent manner, overexpression of CIB1 increased adhesion-induced activation of PAK1 at all time points (for up to 3 hours; Figure 3-2 A). Upon depletion of CIB1, adhesion-induced activation of PAK1 was decreased only at early time points (20 and 45 minutes), but not at later time points (1.5 and 2.5 hours) which were primarily regulated by Rac/Cdc42 (Figure 3-2 B). Moreover, CIB1 overexpression and depletion appeared to significantly affect PAK1-mediated cell migration. In this study, CIB1-induced activation of PAK1 inhibited migration by promoting LIMK-dependent inactivation of the actin regulatory protein cofilin. This is an interesting finding, since PAK1 is also known to increase migration through its downstream activation of MAPK signaling.

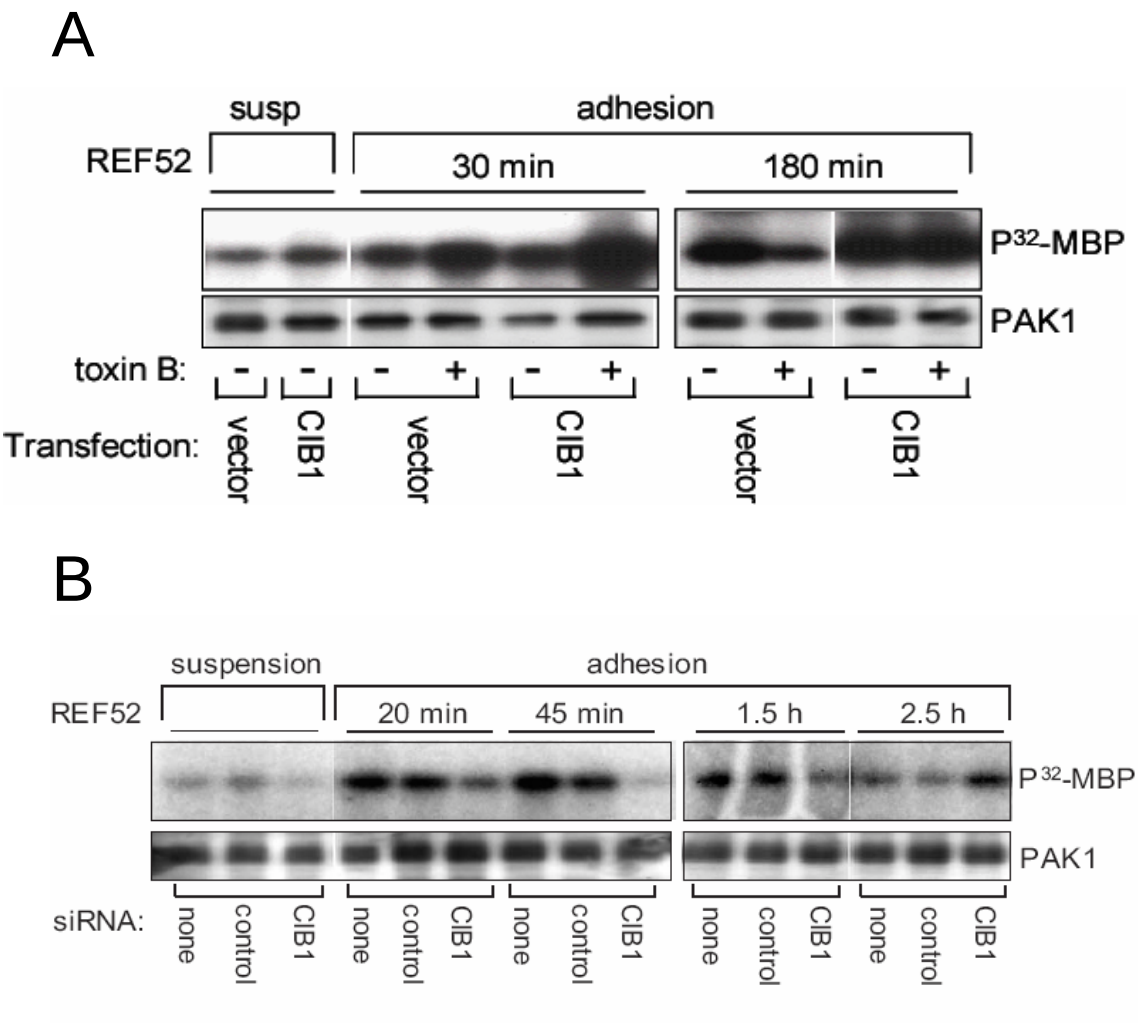

Figure 3-2. CIB1 regulates PAK1 activation in REF52 fibroblasts. (A) CIB1 overexpression increases PAK1 activation in a Rac/Cdc42-independent manner (in the presence of toxin B). **(B)** Knockdown of CIB1 decreases PAK1 activation at early time points (20 and 45 minutes) but not later time points (2.5 hours). **The panels in this figure are published in the following reference: Leisner, T.M., Liu, M., Jaffer, Z.M., Chernoff, J., and Parise, L.V. "Essential role of CIB1 in regulating PAK1 activaiton and cell migration."** ***JCB*****, 2005. 170: 3, 465-476. They are reprinted with permission from The Rockefeller Unviersity Press.**

3.3.5 Integrins

Although the initial yeast-two hybrid classification of CIB1 suggested that it specifically bound to the αIIb cytoplasmic tail, and did not bind to any other integrin cytoplasmic tails (αv, α2, α5, β1, and β3)(5), recent NMR and ITC studies have revealed an association between CIB1 and α5 (Jun Qin, Holly Gentry, and Leslie Parise, unpublished data). Furthermore, binding studies confirmed that αIIb contains a minimal binding sequence for CIB1 that extends beyond the cytoplasmic domain and into the αIIb putative transmembrane region(6;7). This is important since only the cytoplasmic compartments of the αv, α2, and α5 integrins were used in the initial screen(5). This prompted further studies using ITC, which showed that in addition to αIIb, CIB1 can in fact bind to other integrin α chains, including α2, α4, α5, αL, and αM. All interactions between CIB1 and α integrins peptides were found to be of high affinity, with only αM occurring at a slightly lower affinity (binding of CIB1 to αv is still under investigation; Holly Gentry and Yi Wu, unpublished data). These findings therefore suggest that CIB1 may be a global regulator of various types of α integrins, including ones that are critical for vascular homeostasis and angiogenesis. However still to be determined is whether CIB1 binding to these integrin subunits can affect their activation states and contribute to biological functions *in vivo*.

3.4 Role of CIB1 in megakaryocytes and platelets

When the αIIb integrin cytoplasmic domain or its cytoplasmic GFFKR motif (which partly comprises the CIB1 minimal binding sequence) are missing the αIIbβ3 integrin

becomes constitutively active(26). Thus it was hypothesized that since CIB1 directly bound to the cytoplasmic domain of αIIb, it may be important for regulating αIIb activation and downstream signaling. This mode of regulation has been shown to occur for multiple integrins via the β3 integrin subunit(27). Naik and Naik provided the first evidence to demonstrate that CIB1 can functionally regulate platelet spreading and lamellipodia formation through its activation of FAK(9). To validate and build upon these findings, Yuan et al. demonstrated that CIB1 competes with talin for αIIb integrin binding in order to regulate the activation state of αIIb in megakaryocytes(28). Specifically, overexpression of CIB1 dramatically inhibited agonist-induced αIIb activation and binding to fibrinogen. While, in the converse experiment knockdown of CIB1 using RNAi demonstrated an increase in agonist-induced activation. These findings suggested that in physiological situations, CIB1 can maintain the αIIbβ3 integrin in an inactive state, thus regulating platelet aggregation and hemostasis.

To further clarify the role of CIB1 in platelet function and vascular biology a CIB1-KO mouse was generated(29). Despite an anticipated defect in platelet hemostasis, so far CIB1-KO mouse platelets appear to behave normally. In fact, besides a sterility defect in CIB1-KO mice due to a disruption of the haploid phase of spermatogenesis(29), CIB1-KO mice reach all developmental milestones and progress to adulthood without any overt hematological or vascular phenotypes. An explanation for this apparent discrepancy between *in vitro* and *in vivo* findings is yet to be proposed. One probable explanation may be that molecular redundancies in megakaryocytes and platelets are compensating for the chronic

loss of CIB1 in CIB1-KO platelets. Likely candidates that may compensate for the loss of CIB1 may include its newly identified homologs CIB3 and CIB4(11).

3.5 Hypothesized role of CIB1 in ECs and Angiogenesis

Beyond the role of CIB1 in megakaryocytes, platelets, and the testis, little else is know about the biological functions of CIB1. Moreover, the potential role of CIB1 in other vascular tissue, in addition to platelets, has never been explored. In an initial screen, we have found that CIB1 is expressed in different embryonic mouse vasculature structures and various types of ECs (see Chapter 4, Figure 4-1). Since CIB1 also binds and regulate the activity of important angiogenic kinases like FAK(9) and PAK1(24), we hypothesized that CIB1 may play an important role in EC signaling and function. Furthermore, although CIB1-KO mice demonstrate no obvious defect in vascular development, we postulated that pathological forms of angiogenesis in CIB1-KO mice may be altered.

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CHAPTER 4

CIB1 Regulates EC Signaling and Function

4.1 Abstract

CIB1 is a 22kDa, EF-hand containing, regulatory protein that was originally identified to bind the platelet-specific integrin αIIb. Subsequently, CIB1 was found to bind various other proteins, including proteins that can regulate EC function. However, the role of CIB1 in vascular tissue has never been explored. We found that CIB1 mRNA expression is highest in vascularized organs such as the testis, bone marrow, kidney, placenta, and the embryonic yolk sac. CIB1 is also expressed in various species and types of ECs, including microvascular and macrovascular ECs. We demonstrate here that when CIB1 is depleted from ECs by homologous recombination (CIB1-KO ECs), we observe attenuated adhesion-induced PAK1 activation and ERK1/2 phosphorylation. CIB1-KO ECs also have a significant decrease in growth factor-induced MMP2 expression and secretion. Moreover, CIB1-depleted ECs demonstrate significantly decreased EC functions, including decreased migration, proliferation, tubule formation, and monolayer resistance. Treatment with growth factors, such as VEGF or bFGF, did not rescue defects observed in CIB1-depleted ECs, but re-introduction of CIB1 in CIB1-KO ECs did restore haptotatic migration to normal levels. Thus, collectively our findings demonstrate that CIB1 can critically regulate various EC functions and is necessary for proper EC signaling. This work identifies a previously unknown role for CIB1 in the vasculature, and suggests that it may have a role in angiogenesis.

4.2 Introduction

CIB1, a 22kDa EF-hand-containing protein, was originally identified in a yeast-two-hybrid screen as a binding partner for the cytoplasmic tail of the platelet integrin αIIb(2). *In vitro*, CIB1 was found to compete with the cytoplasmic protein talin for binding of αIIb, and inhibit agonist-induced αIIbβ3 activation in megakaryocytes(3). However, CIB1 is widely distributed, suggesting that it has roles in other cell types besides megakaryocytes, and contributes to cellular functions that are independent of αIIbβ3(4;5). Thus far, CIB1 has been shown to associate with proteins such as the Alzheimer's disease associated presenilin-2(6), $InsP_3R$(6;7), FAK(8), and PAK1(9). Among these binding partners, FAK and PAK1 have been shown in multiple studies to regulate EC signaling and function, thus prompting us to determine whether CIB1 has a role in ECs(10-12).

The endothelium, which is comprised of a cohesive single-cell layer of ECs, covers a large surface area (4000-7000 m^2 in an average sized human) and forms a metabolically active interface between blood and tissue(13). Accordingly, ECs are pivotal in physiological processes such as controlling vasomotor tone, trafficking of cells and nutrients, maintaining blood fluidity, regulating permeability, and facilitating new blood vessel formation in healing and regenerating tissue(14). Just as important however, dysfunction or aberrant activation of ECs can lead to a diverse set of diseases (such as atherosclerosis, thrombosis, or acute and chronic inflammation) that span various clinical disciplines including cardiology, pulmonology, infectious diseases, and oncology(15).

The formation of new blood vessels from pre-existing vasculature is an EC dependent biological process that is referred to as angiogenesis, and occurs in both physiological and pathological situations(16). During the activation phase of angiogenesis, in response to various stimuli (i.e. hypoxia or inflammation), angiogenic growth factors (such as VEGF of bFGF) are typically secreted by stromal cells or liberated from the ECM through the action of ECM degrading MMPs(17;18). In turn, these growth factors bind to their cognate receptors on the surface of ECs, activate intra-endothelial signaling cascades, induce expression of various genes, and ultimately alter various EC functions(19). For example, growth factors can first stimulate ECs to undergo a remarkable increase in proliferation. If a gradient has been established by the secreted growth factors, then newly formed ECs will also participate in directed migration toward areas of higher growth factor concentrations(20). Migrating cells will also form a trail of ECs that can eventually envelop upon one another to form nascent tubular structures. During the resolution phase of angiogenesis, these nascent tubules become remodeled into mature blood vessels that can facilitate blood flow(21).

In recent years, much has been learned about the molecular regulation of EC angiogenic functions. However, endothelial angiogenic signaling is highly complex and the molecular mechanisms that regulate ECs are still not fully elucidated. In this study we demonstrate that in ECs, CIB1 regulates the activity of the angiogenic kinase PAK1, and affects downstream signaling events. Furthermore, we also demonstrate that CIB1 is essential for proper EC functions such as migration, proliferation, tubule formation, and monolayer resistance. Thus, we describe here the novel regulatory role of CIB1 in ECs,

which establishes the bases for later studies investigating the role of CIB1 in angiogenesis, *in vivo*.

4.3 Methods

Reagents, lysate preparation, and Western blotting. MECs and BAECs were maintained in culture using high-glucose DMEM (Sigma, St. Louis, MO) supplemented with 3% fetal bovine serum (FBS; Sigma). HUVECs, HPAECs, and HCAECs were maintained in EBM culture media according to manufacture's instructions (Cambrex, East Rutherford, NJ). MHECs and MLECs were isolated in isolation media (high-glucose DMEM with 20% FBS, 20 U/mL penicillin/streptomycin antibiotic (Gibco, Carlsbad, CA), and 20 U/L Heparin (Sigma)) and were maintained in highly supplemented growth media (isolation media supplemented with 0.5X non-essential amino acids (Gibco), 0.5X sodium pyruvate (Gibco), 0.5X L-glutamate (Gibco), 25 mM HEPES (Cellgro, Lawrence, KS), and 100 μg/mL endothelial cell mitogen (ECGS; Biomedical Technologies, Stoughton, MA)). Cells were serum starved in basal media (high-glucose DMEM supplemented with 0.1% BSA and 20 U/L heparin). For lysis of all EC types, 5 X 10^5 cells were pelleted and lysed in modified CHAPS buffer (20 mM Hepes, pH 7.4, 0.15 M NaCl, 10 mM CHAPS, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM each of $CaCl_2$ and $MgCl_2$, and Protease Inhibitors Cocktail Set III (Calbiochem, San Diego, CA)) on ice for at least 30 minutes, and lysates were collected following centrifugation. Total protein samples were then separated by SDS-PAGE, transferred to a PVDF membrane, and subjected to Western blotting. As described previously(9;22), proteins were detected with CIB1 chicken polyclonal antibody, NH_2-

terminal-specific anti-PAK1 polyclonal antibody (Santa Cruz Biotechnology, Inc.), and anti-total and anti-phospho-ERK1/2 polyclonal antibodies (Cell Signaling, Inc.). Total MMP2 was detected using anti-MMP2 specific goat IgG primary antibody (R&D Systems, Minneopolis, MN), and goat IgG secondary antibody (Sigma).

Northern blotting. Nitrocellulose blots of total adult mouse organ RNA and embryonic RNA were a generous gift from James Ferguson and Cam Patterson, UNC-CH. A full-length CIB1 probe was constructed using Prime It Random kit (Stratagene, La Jolla, CA). A CIB1 cDNA probe was labeled with ^{32}P-dCTP and hybridized to nitrocellulose blots.

Short hairpin RNA construction and transduction. A lentiviral expression system was adopted as previously described(1). For CIB1 knockdown, human (5'accggagcgaatctgcatggtcttcaagagagaccatgcagattcgctccttttttc3') and mouse (5'accgtgcccttcgagcagattcttcaagagagaatctgctcgaagggcacttttttc3') CIB1 shRNA were cloned into a mammalian FG12 expression vector that separately expresses green florescent protein (GFP) via a separate promoter. For control shRNA in murine ECs, human CIB1 shRNA was used since as previously reported(3), it has no homology to murine CIB1 or to any sequence in the mouse genome. For control shRNA in human ECs, mouse R-Ras shRNA (5' accgctcttcacacagatcctcttcaagagagaggatctgtgtgaagagctttttc 3') was used since it has no homology to any sequence in the human genome. For CIB1 overexpression, the lentiviral vector was reconstructed to express CIB1 cDNA downstream of a cytomegalovirus (CMV) promoter. A control cDNA vector was also constructed containing only the CMV promoter. HEK293 cells were transfected with lentivirus packaging vectors, as well as shRNA or the

cDNA construct of interest. ECs were transduced with lentivirus collected from HEK293 culture supernatant at multiplicity of infection (MOI) 10 – 25. CIB1 knockdown or overexpression was confirmed via Western blotting. Densitometry with the software Quantity One (Fluor-S Multimager; Bio-Rad Laboratories, Hercules, CA) was used to determine fold change in CIB1 expression.

Isolation of primary endothelial cells. Hearts and lungs of approximately 1-3 month old WT and CIB1-KO mice were dissected from the mouse mediastinum. Organs were gently minced, collagenase-digested, and strained. The resulting cell suspension underwent positive cell sorting using PECAM-1 (BD Biosciences, San Jose, CA) anti-rat IgG-conjugated magnetic beads (Invitrogen, Carlsbad, CA). Isolated cells were plated in tissue culture flasks coated with 5ng/mL fibronectin and cultured in growth media. Heart and lung ECs were then purified with a second round of positive cell sorting using ICAM-2 (BD Biosciences) coated magnetic beads. The remaining ECs were cultured for up to 3 passages.

PAK1 kinase activation assay. As previously described(9), serum starved ECs were held in suspension or seeded onto tissue culture plates coated with 10μg/mL fibronectin. Lysates were prepared by addition of cold lysis buffer supplemented with 10% glycerol and 0.2mM pervanadate. Equal amounts of lysate protein were subjected to SDS-PAGE and Western blotted for total and phospho-ERK1/2. Lysates were also subjected to PAK1 immunoprecipitation, and PAK1 kinase activity was assayed using myelin basic protein as substrate. For each condition, experiments were repeated at least 3 times.

Gelatin zymography. The activity of MMP2 in conditioned medium was determined by gelatin zymography. Briefly, ECs were serum starved for at least 24h, followed by addition of phenol-free high-glucose DMEM media supplemented with 100ng/mL of VEGF or bFGF (R&D Systems, Minneopolis, MN), or 160ng/mL of PMA. Conditioned media was collected 18h later, filtered through 0.45μm Whatman filters, and concentrated using Vivaspin 10kDa concentrator (Sartorius, Edgewood, NY). Equal protein concentrations were loaded under nonreducing conditions onto pre-made 10% zymograms and treated with renaturation and development buffers according to manufacture's instructions (Biorad, Hercules, CA). For each condition, experiments were repeated at least 3 times.

Boyden chamber haptotactic assay. Haptotactic migration was performed using 8μm pore polycarbonate transwell membranes (Corning, Corning, NY) coated underneath with 25μg/mL fibronectin (Calbiochem). Cells were serum starved for at least 24h, and 2 X 10^5 cells were added to the top chamber. Cells migrated in basal media (0.1% BSA and 20U heparin) supplemented with or without 250ng/mL bFGF or VEGF. After 6h, non-migrating cells were removed from the topside of the membrane, and membranes were isolated, fixed, and stained with Alexa Fluor 594-conjugated phalloidin (1:1000; Invitrogen). To obtain counts of migrating cells expressing pre-transduced lentiviral vectors, both Texas red and GFP fluorescence images of at least 5 random digital frames were collected using a Nikon Eclipse TE300 inverted florescent microscope equipped with a CoolSnap Photometrics HQ camera from Media Cybernetics with Image-Pro Plus 5.0 software. Images were transferred

to NIH ImageJ software (http://rsb.info.nih.gov/ij/), where total numbers of haptotactic cells were counted and reported as mean number of cells per imaged field. For each condition, experiments were repeated at least 3 times.

Monolayer wound healing culture assay. EC cultures were grown to confluency in 10 cm culture plates. Cells were serum starved for at least 48 h and wounds were created in the monolayer culture with a medium sized pipette tip. Growth media was then added and images were collected at approximately the same areas of the same wounds 0, 16, 20, and 24h post-wound induction. Percentage in wound area closure was measured using ImageJ software.

Matrigel tubule formation assay. Using a 48-well culture format, wells were coated with 150μL of GFR Matrigel (BD Biosciences). Serum starved 5 x 10^4 cells were added on top of the polymerized GFR Matrigel. Cells were then incubated in the presence of growth media, or basal media supplemented with 25ng/mL of bFGF or VEGF. Images were collected 18 hours after incubation. For tubules formed by ECs that had been pre-transduced with lentiviral vector, fluorescent images were also collected to confirm EC transduction efficiency. Tubule number was analyzed from collected images using ImageJ software. For each condition, experiments were repeated at least 3 times.

ELISA. For bromodeoxyuridine (BrdU) incorporation, 1 X 10^4 ECs were adhered in a 96-well culture format followed by serum starvation for at least 24h. Growth media or basal media supplemented with 25ng/mL bFGF or VEGF was then added. BrdU addition

and detection at 24h were performed according to manufacturer's instructions (Roche, Basel, Switzerland). For each condition, experiments were repeated at least 3 times and absorbance measured in a multi-well spectrophotometer at 450nm (Spetra MaxPlus, Molecular Devices, Sunnyvale, CA).

Monolayer real-time cell electronic sensing (RT-CES). Isolated ECs were cultured to 60% confluency and starved overnight in basal media. ECs ($2.5x10^4$ cells) were seeded in fibronenctin-coated (5ug/mL) electronic microtiter plates (E-Plate, ACEA Biosciences Inc., San Diego, CA) and supplemented with growth media. EC adhesion and ionic resistance across forming monolayers were monitored using a computerized RT-CES apparatus (ACEA Biosciences, Inc.). For each cell type the assay was performed in replicates of 8.

Statistical analysis. We compared continuous variables with the Student's t-test. We considered $P < 0.05$ to be significant.

4.4 Results

4.4.1 CIB1 is expressed in vascular structures and ECs.

Northern blot using full-length CIB1 cDNA probe confirms CIB1 mRNA expression in adult mouse lung, kidney, and testis. Additionally, we found that CIB1 mRNA is expressed in mouse ovary, spleen, and bone marrow. CIB1 mRNA expression was highest in the testis, kidney, and bone marrow (Figure 4-1A). Northern blotting also confirmed CIB1

mRNA expression in mouse embryonic tissue. Specifically, we found that it is expressed at embryonic day (ED) 7.5, 8.5, and 9.5. CIB1 mRNA was highly expressed in the primitive embryonic vascular structures such as the yolk sac and placenta of ED 8.5 and 9.5 mouse embryos (Figure 4-1B). Furthermore, Western blot analysis reveled that CIB1 is expressed in various types of ECs including transformed (mouse intraembryonic endothelial cells; MECs(23), and bovine aortic endothelial cells; BAECs), primary (human aortic endothelial cells; HAECs, HUVECs, human coronary artery endothelial cells; HCAECs, and human pulmonary artery endothelial cells; HPAECs), embryonic (MECs), macrovascular (BAECs and HAECs), and microvascular ECs (HUVECs, HCAECs, and HPAECs; Figure 4-1C).

4.4.2 Lentiviral mediated knockdown and overexpression of CIB1.

Lentiviral transduction is more efficient than standard transfection techniques, and is a reliable method of gene transfer. In order to test the role of CIB1 in EC signaling and function, we adopted this technique to stably knockdown and overexpress CIB1 in ECs. To knockdown CIB1, cells were transduced with a vector that expresses shRNA specific for CIB1. For overexpression, a vector that expresses CIB1 cDNA was instead used. Figure 4-2 illustrates a schematic of the CIB1 knockdown and overexpression vectors that were used as well as their respective controls. At reasonable viral titers, lentiviral transduction efficiency

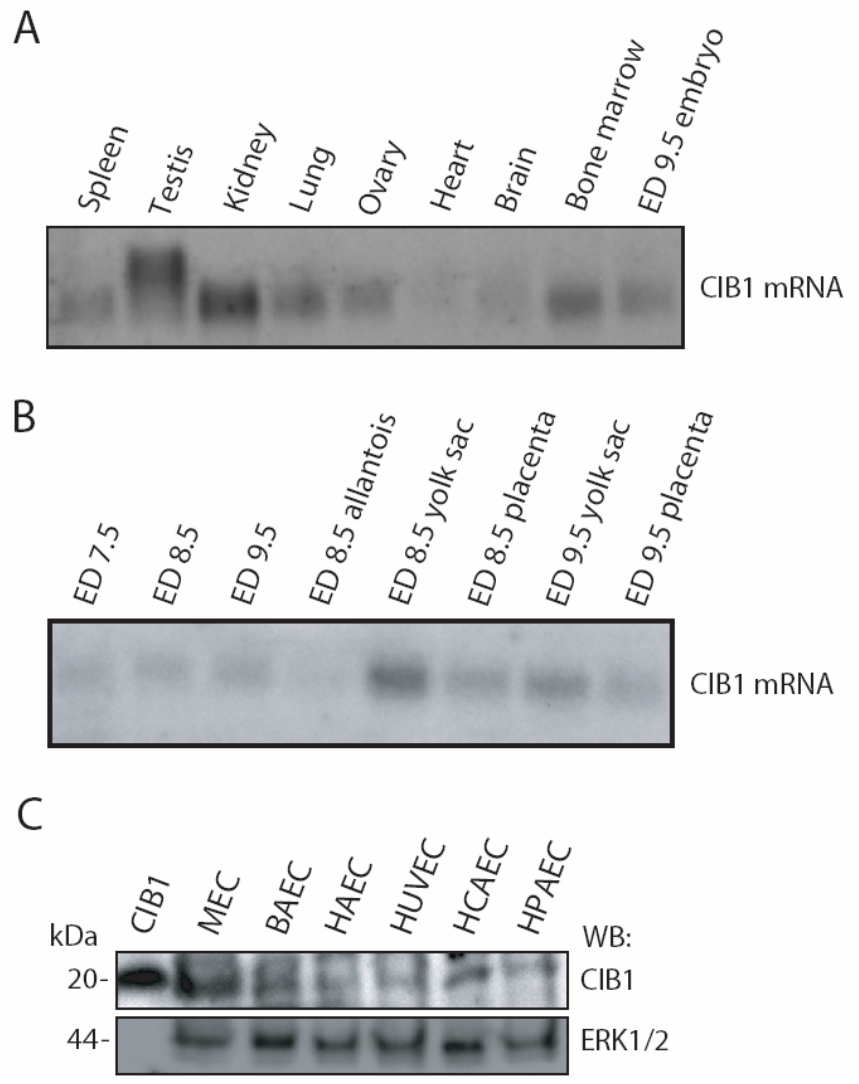

Figure 4-1. CIB1 is expressed in various organs, mouse embryonic vascular structures, and EC types. (A) Northern blot using full-length CIB1 cDNA probe demonstrates CIB1 mRNA expression in various adult mouse organs. CIB1 mRNA expression is highest in the testis, kidney, and bone marrow. **(B)** Northern blot also demonstrates CIB1 mRNA expression in mouse embryonic tissue at ED 7.5, 8.5, and 9.5. CIB1 mRNA is highly expressed in primitive embryonic vascular structures such as the yolk sac and placenta of ED 8.5 and 9.5 mouse embryos. For panels **(A)** and **(B)**, 28S rRNA loading equivalency does not account for differences in tissue expression of CIB1 mRNA (results not shown). **(C)** Relative to 1ng of CIB1 (first lane), Western blot reveled that CIB1 is expressed in various types of ECs, including mouse, bovine, human, embryonic, macrovascular, and microvascular ECs. MEC, mouse intraembryonic EC; BAEC, bovine aortic EC; HAEC, human aortic EC; HUVEC, human umbilical vein EC; HCAEC, human coronary artery EC; HPAEC, human pulmonary artery EC.

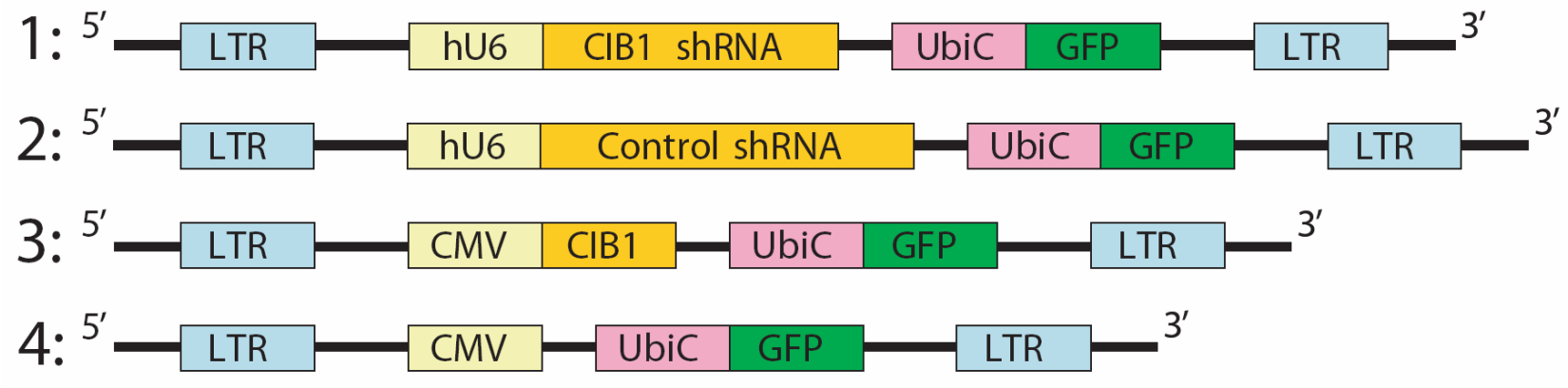

Figure 4-2. Lentiviral gene delivery system for CIB1 knockdown and overexpression. LTR-flanked double-cassette FG-12 backbone vectors obtained from D. Baltimore(1) were modified for CIB1 knockdown and overexpression. For CIB1 knockdown, lentiviral vector was packaged with a FG12 vector expressing either control shRNA or CIB1 shRNA via a polymerase III hU6 promoter, and GFP via a separate UbiC promoter (vectors 1 & 2). For CIB1 overexpression and empty vector control, similar double-cassette vectors were used to either express or not express CIB1 cDNA via a CMV promoter. LTR, long terminal repeat; hU6, human U6 promoter; shRNA, short-hairpin RNA; UbiC, Ubiquitin C promoter; GFP, green fluorescent protein; CMV, cytomegalovirus promoter.

was >95% in different EC cell types such as MECs and HUVECs, as determined by GFP expression (Figures 4-3A & 4-3B). Western blot analysis confirmed stable knockdown by up to 80%, and overexpression by up to 600% (Figures 4-3C & 4-3D).

4.4.3 PAK1 and ERK1/2 activation in ECs is disrupted upon the loss of CIB1.

Leisner et al. previously determined that CIB1 regulates PAK1 activation in fibroblasts and epithelial cells (see Chapter 3; Figure 3-2)(9). Since various studies have demonstrated that proper PAK1 activation and signaling is essential for EC function and angiogenesis(10;11;24), we asked whether the loss of CIB1 in ECs can also alter PAK1 activation. We tested this in mouse lung ECs (MLECs) isolated from WT and CIB1-KO mice, and preliminarily in transduced MECs. In agreement with previous findings, *in vitro* kinase assays demonstrated significantly decreased PAK1 activation in CIB1-KO ECs shortly after adhesion to fibronectin (20 and 45 minutes; Figure 4-4A). At 180 minutes after adhesion, PAK1 activation in both WT and CIB1-KO ECs appeared to subside to minimal levels. Both WT and CIB1-KO ECs also demonstrated minimal PAK1 activation when held in suspension, as demonstrated in other cell types. Although MECs transduced with control shRNA or CIB1 shRNA did not demonstrate a decrease in PAK1 activation during suspension, we preliminarily observed a similar decrease in PAK1 activation at 20 and 45 minutes after adhesion of cells (Figure 4-4B). By 180 minutes, both cell types again demonstrated minimal PAK1 activation.

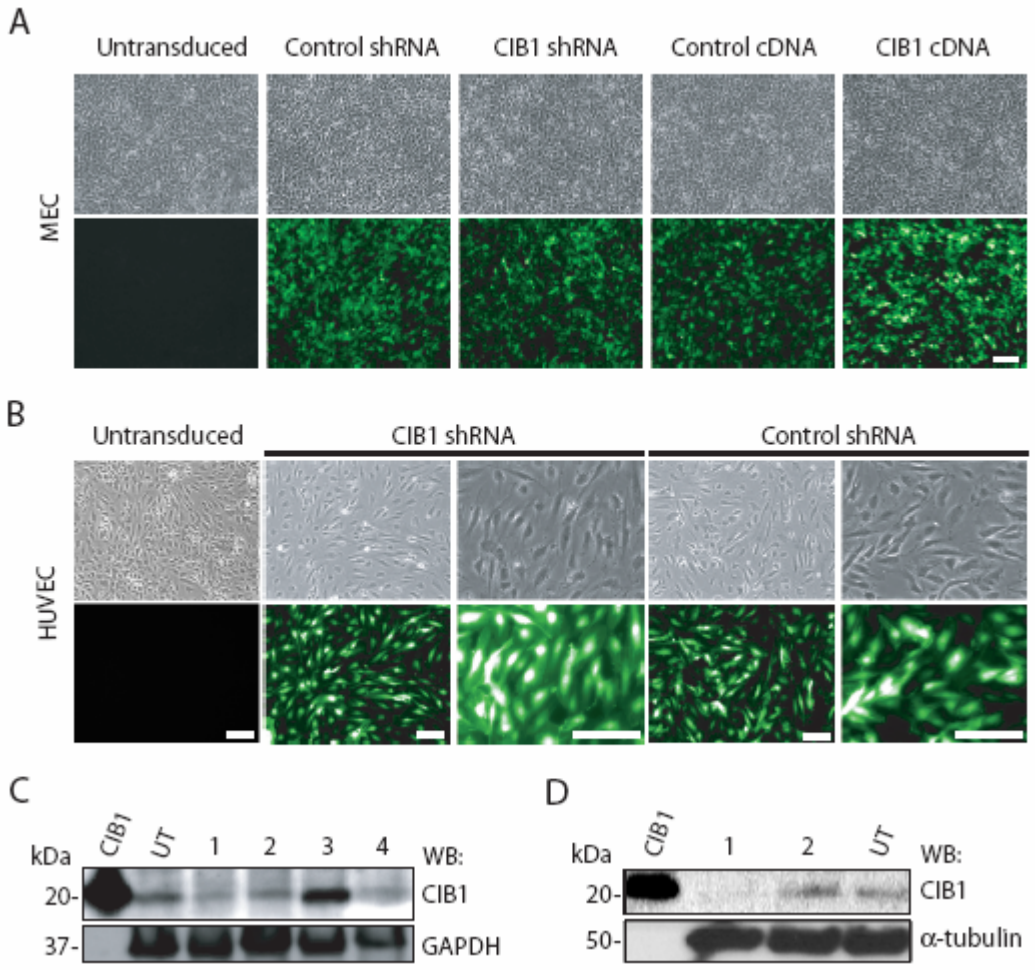

Figure 4-3. Efficient lentiviral transduction of MECs and HUVECs. (A) Confluent monolayers of MECs were transduced with lentivirus packaging one of four different vectors (CIB1 shRNA (vector 1), control shRNA (vector 2), CIB1 cDNA (vector 3), or control cDNA (vector4)). Three days after infection, GFP expression was detected in approximately 95% of cells. **(B)** Similarly, HUVECs were transduced with lentivirus packaging either CIB1 shRNA (vector 1) or control shRNA (vector 2). At high and low magnifications, approximately 100% of cells express GFP 3 days after infection. Untransduced MECs and HUVECs do not express GFP, **(A** and **B)**. Short scale bars; 40μm, and long scale bars; 50μm. **(C** and **D)** Relative to 5ng of CIB1, stable knockdown and overexpression in ECs was confirmed via Western blot. In MECs and HUVECs, stable CIB1 knockdown was up to 80% and 90%, respectively, and CIB1 overexpression in MECs was up to 600%.

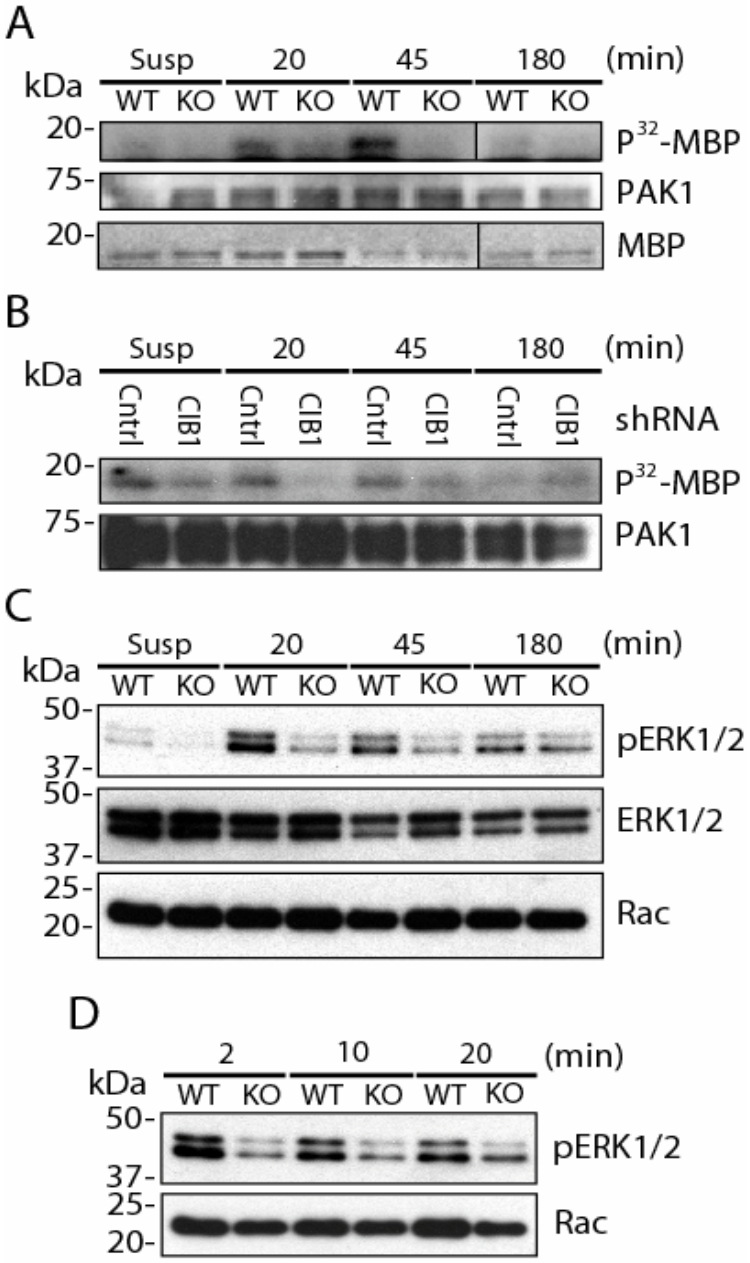

Figure 4-4. Loss of CIB1 disrupts PAK1 activation and ERK1/2 phosphorylation. (**A**) PAK1 activity in WT and CIB1-KO MLECs upon adhesion to fibronectin for 20, 45, and 180 minutes, as demonstrated by *in vitro* myelin basic protein phosphorylation ([^{32}P]MBP). Western blots of total PAK1 protein and coomassie stain of MBP serve as loading controls. (**B**) PAK1 activity in control and CIB1-depleted MECs following adhesion. Western blot of PAK1 protein serves as loading control. **(C)** ERK1/2 phosphorylation as detected with a phospho-ERK1/2 specific antibody, in WT and CIB1-KO MLECs held in suspension or following adhesion. Western blots of total ERK1/2 and Rac serve as loading controls. (**D**) ERK1/2 phosphorylation in WT and CIB1-KO MLECs treated with VEGF (25ng/mL) for 2, 10, and 20 minutes. **Blots are contributed by Dr. Tina Lisner**.

Since various studies have underscored the importance of the downstream effectors of PAK1, ERK1/2, in EC angiogenic signaling(10;25-27), we asked whether ERK1/2 activation is also disrupted in ECs upon the loss of CIB1. Although no significant difference was detected in transduced MECs (data not shown), CIB1-KO MLECs demonstrated a significant decrease in ERK1/2 phosphorylation levels compared to WT MLECs, at 20 and 45 minutes after adhesion to fibronectin (Figure 4-4C). Similar to the kinetics observed for PAK1 activation, no difference in ERK1/2 phosphorylation was detected at later time points (180 minutes) after adhesion. The decrease in ERK1/2 phosphorylation appeared to be unique since we did not detect any differences in the phosphorylated protein levels of Akt, p38, and protein kinase C (PKC; data not shown). Furthermore, decreased ERK1/2 activation was also preliminarily observed in CIB1-KO ECs treated with VEGF for 2 and 10 minutes (Figure 4-4D).

4.4.4 Loss of CIB1 in ECs decreases MMP2 expression.

Numerous signaling cascades can become activated downstream of PAK1 (for review see(28)). Among them, MAPKs are implicated in the production of MMPs(29;30). Since the loss of CIB1 in ECs decreases responsiveness to angiogenic growth factors and leads to decreased PAK1 and ERK1/2 activation upon fibronectin adhesion, we asked whether MMP expression was also disrupted. Enzymatic activity of MMPs was assessed by gelatin zymography, which revealed increased lytic zones only at the molecular mass of active (68kDa) and latent form of MMP2 (gelatinase A; faint band at 72kDa) in the conditioned media of growth factor-treated WT MLECs, but not CIB1-KO MLECs (Figure 4-5A).

Western blotting also demonstrated less growth factor-induced MMP2 expression in CIB1-KO MLEC conditioned media (Figure 4-5B), and decreased MMP2 protein expression in CIB1-KO mouse heart ECs (MHECs; Figure 4-5C). However, MMP9 (gelatinase B) secretion was undetectable in both WT and CIB1-KO ECs (data not shown). Furthermore, no differences in the expression of the physiological activator and inhibitor of MMP2 (tissue inhibitor of metalloproteinases-2; TIMP-2, and MMP14; MT1-MMP, respectively(31;32)) were observed between both cell types (data not shown).

4.4.5 Loss of CIB1 decreases EC migration.

Disrupted PAK1 and ERK1/2 signaling may significantly affect EC function. Since both CIB1 and PAK1 have been implicated in cellular migration, we first tested the role of CIB1 in EC migration. Knockdown of CIB1 with short hairpin (sh) RNA decreased haptotaxis of MECs on fibronectin (38% relative to control MECs; Figure 4-6A). Similarly, deletion of CIB1 by homologous recombination in CIB1-KO MLECs resulted in a similar decrease (24% decrease relative to WT and CIB1 heterozygous MLECs; Figure 4-6B). While angiogenic growth factors bFGF and VEGF increased haptotaxis in untransduced MECs (data not shown) and control MECs, they did not stimulate haptotaxis to the same level in CIB1 knockdown MECs (Figure 4-6A). Interestingly, CIB1 overexpression in MECs did not elevate haptotaxis beyond control levels, suggesting that endogenous CIB1 is expressed maximally for this effect (data not shown). Finally, re-introduction of CIB1 in CIB1-KO MLECs restored haptotatic migration to normal levels (Figure 4-6B). These results suggest

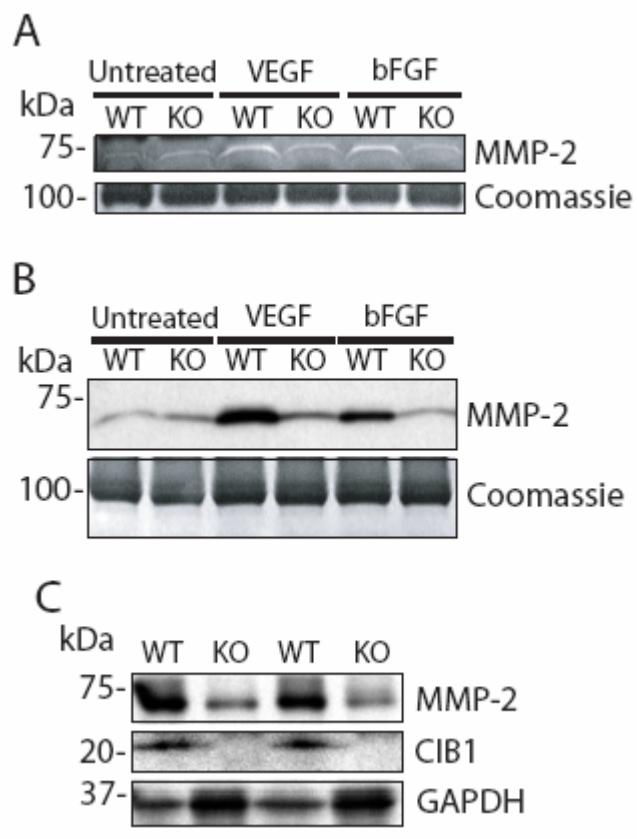

Figure 4-5. Loss of CIB1 decreases MMP-2 secretion and expression. (A) Conditioned media of growth factor-treated and untreated WT and CIB1-KO MLECs was subjected to gelatin zymography. Clear bands in de-stained gel represent MMP-2. Coomassie stained non-specific bands serve as a loading controls. (**B**) Western blot of MMP-2 in conditioned media of growth factor-treated and untreated WT and CIB1-KO MLECs. Coomassie stained non-specific bands serve as a loading controls. (**C**) Western blot of total MMP-2 and CIB1 protein in duplicate WT and CIB1-KO MHEC lysates. Blot for GAPDH serves as a loading control. **Panel (C) is contributed by Dr. Weiping Yuan**.

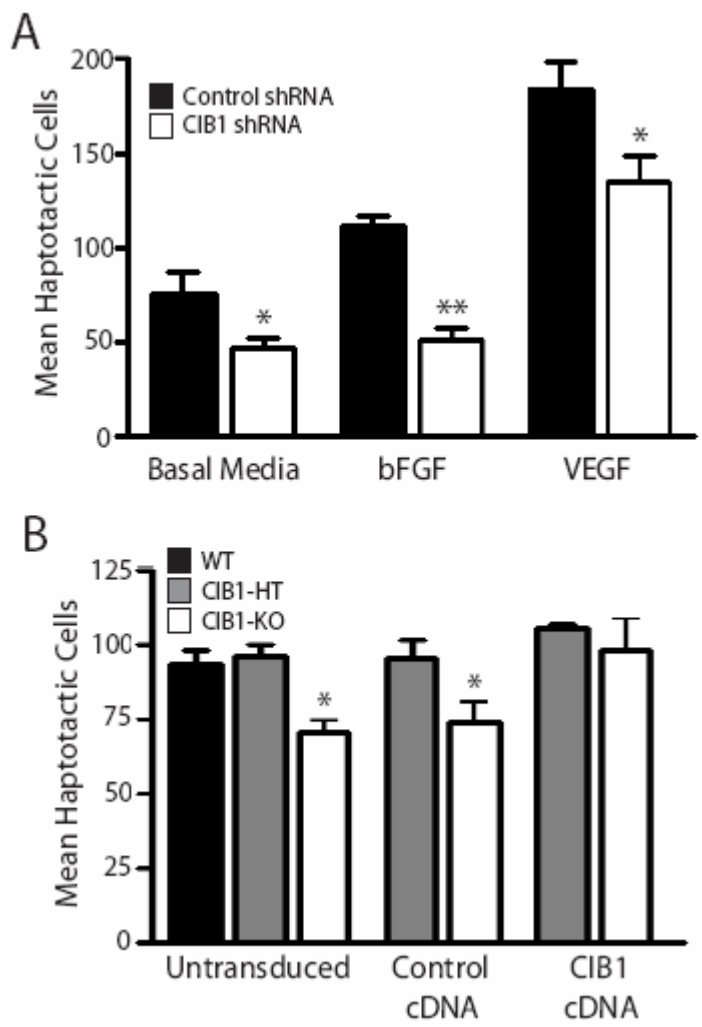

Figure 4-6. Loss of CIB1 decreases EC haptotatic migration. Loss of CIB1 decreases EC function. **(A)** Quantification of mean number of MECs transduced with CIB1 shRNA or control shRNA that migrated across fibronectin in a Boyden chamber in the presence or absence of bFGF or VEGF. **(B)** Mean value of haptotactic cells in transduced or untransduced, WT, CIB1 heterozygous, and CIB1-KO MLECs. CIB1 heterozygous and CIB1-KO MLECs were transduced with either control cDNA or CIB1 cDNA vectors. Error bars represent SEM (n = at least 3 for all conditions); * $P < 0.05$, ** $P < 0.001$.

that CIB1 is necessary for both growth factor-stimulated and unstimulated haptotactic EC migration.

To further confirm these findings, we tested migration using a cell-culture monolayer wound healing assay(33). Monolayer wounds in both CIB1 knockdown MEC and CIB1-KO MHEC resolved less rapidly, relative to control MECs and WT MHECs, respectively (Figures 4-7A & 4-7B). These data provide further evidence that CIB1 expression is necessary for normal EC migratory behavior.

4.4.6 Loss of CIB1 decreases EC proliferation.

Proliferation of ECs is fundamental to angiogenesis (16). Relative to control, MECs depleted of CIB1 by shRNA, and cultured in either growth media or in basal media supplemented with bFGF or VEGF, showed modest yet significantly decreased BrdU incorporation (Figure 4-8A). Consistent with this, CIB1-KO MHECs and MLECs cultured in growth media also demonstrated a 61% and 35% decrease, respectively, in BrdU incorporation compared to WT ECs (Figure 4-8B). Direct cell-counts of cultured CIB1 knockdown MECs and CIB1-KO MHECs further confirmed a significant decrease in proliferation relative to control MECs and WT MHECs (data not shown). Thus, in addition to migration, CIB1 is also necessary for normal rates of EC proliferation.

4.4.7 Loss of CIB1 decreases EC tubule formation.

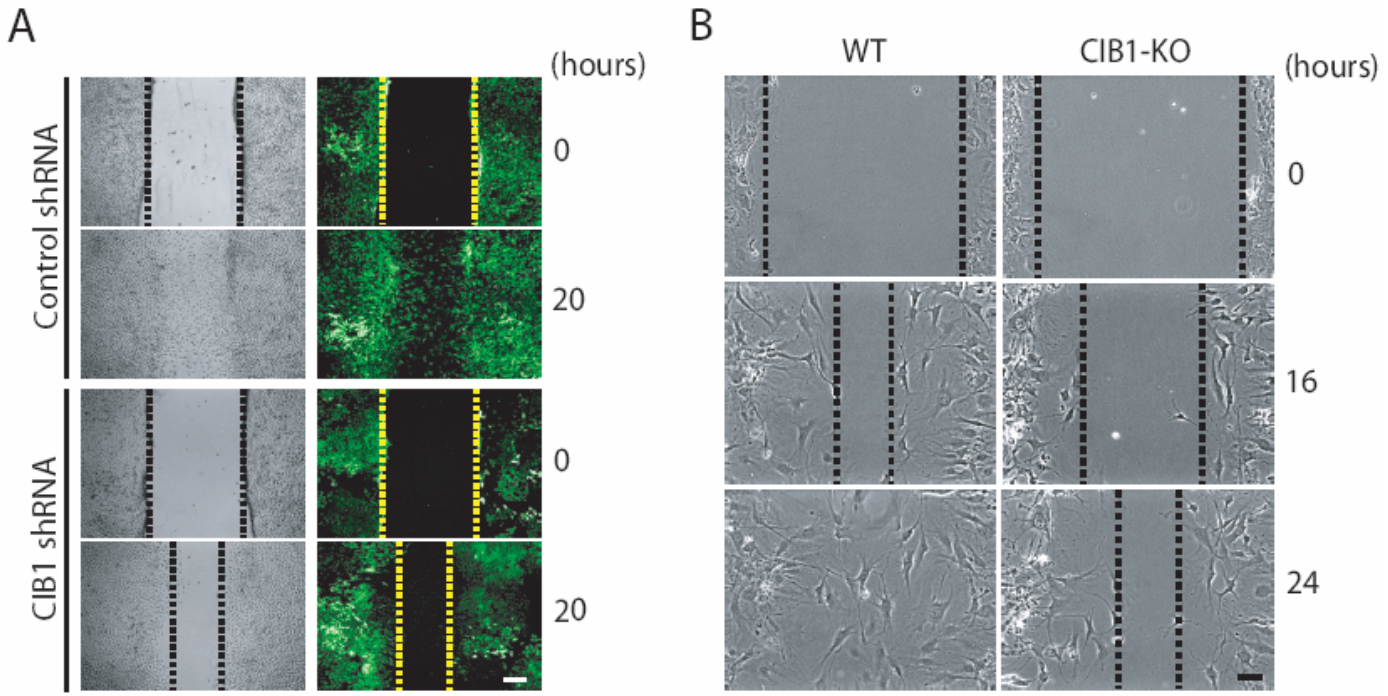

Figure 4-7. Loss of CIB1 decreases EC monolayer wound healing. (A) Differential interference contrast (DIC; left) and GFP (right) fluorescent images of monolayer wounds created in confluent cultures of MECs transduced with control shRNA or CIB1 shRNA. GFP images confirm MEC transduction. Dotted lines outline the wound boundaries. Although monolayer wounds in control MECs completely resolve by 20h post-wound induction, CIB1 knockdown MEC monolayer wounds only resolve by approximately 48%. Scale bar, 100µm. (**B**) Accordingly, monolayer wounds in WT MHECs completely resolved by 24h, whereas CIB1 MHECs only resolve by approximately 64%. Scale bar, 40µm.

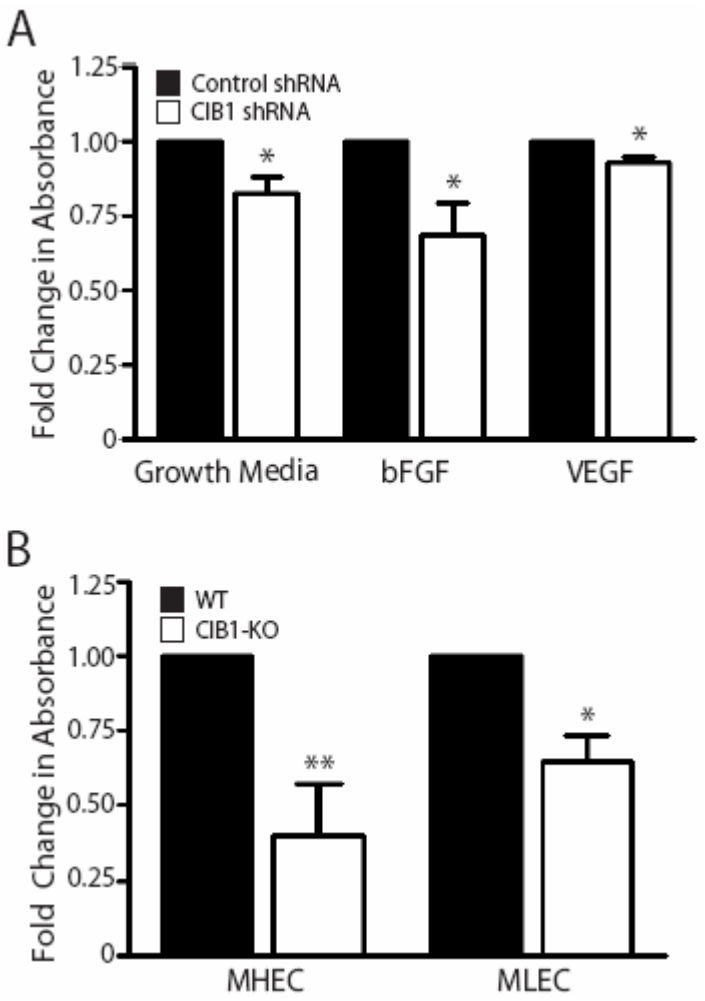

Figure 4-8. Loss of CIB1 decreases EC proliferation. (A) Quantification of BrdU incorporation as the fold difference in absorbance (O.D. 370) in MECs cultured in growth media, or in basal media supplemented with bFGF or VEGF. **(B)** Fold change in BrdU incorporation in CIB1-KO and WT MHECs and MLECs cultured in growth media. Error bars represent SEM (n = at least 3 for all conditions); * $P < 0.05$, ** $P < 0.001$.

To determine whether loss of CIB1 affects other EC functions, we assessed tubule formation on growth factor reduced (GFR) Matrigel. In the presense of growth media, CIB1 knockdown MECs and CIB1-KO MHECs had a 26% and 44% reduction in nascent tubule formation, compared to their respective controls (Figure 4-9A & 4-9B). Treatment of CIB1 knockdown MECs and CIB1-KO MHECs with bFGF or VEGF did not stimulate tubule formation to the same extent as control cells (Figure 4-9A & 4-9B). Hence, in addition to EC migration and proliferation, CIB1 may be required for efficient EC sprouting and invasion that also contribute to angiogenesis.

4.4.8 Decreased monolayer resistance in CIB1-KO ECs

In vivo, ECs inside blood vessels form a semi-permeable barrier that is selective for the transport of nutrients and macromolecules in and out of blood vessels. This process is regulated by various factors including angiogenic growth factors, adhesion molecules, or cytotoxic angents. Since in our pervious assays CIB1 appears to be an important regulator of EC function, we wanted to determine weather it also plays a role in EC barrier function. Using an RT-CES apparatus, we observed that both WT and CIB1-KO ECs reached near maximal adhesion to fibronectin by approximately 1.5 hours (Figure 4-10). However, over a period of nearly 17 hours, we also observed a dramatic and consistent decrease in the ionic resistance generated across the CIB1-KO EC monolayer compared to the WT EC monolayer. This suggests that CIB1 is also critical for EC monolayer integrity – a function that may have serious manifestations *in vivo*.

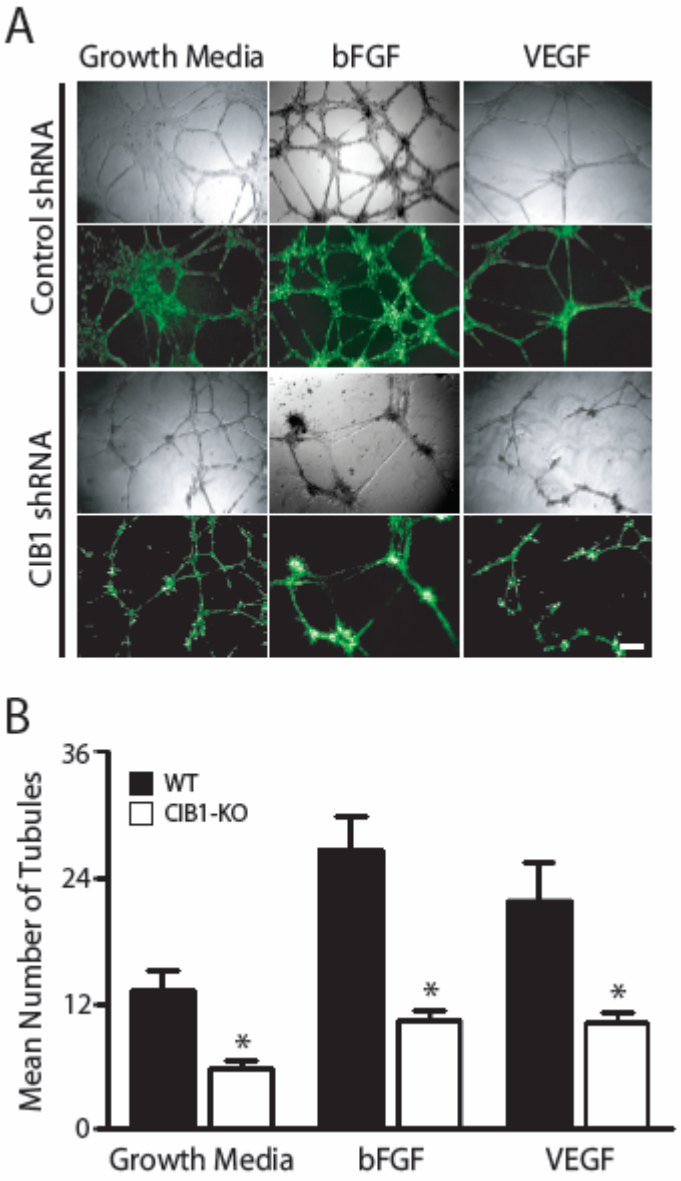

Figure 4-9. Loss of CIB1 decreases EC tubule formation on GFR Matrigel. (**A**) DIC and GFP fluorescent images of CIB1 shRNA transduced MECs and control shRNA transduced MECs following treatment with growth media, bFGF, or VEGF. GFP images confirm MEC transduction and expression of vectors. Scale bars, 100µm. (**B**) Quantification of tubule formation in CIB1-KO and WT MHECs following treatment with growth media, bFGF, or VEGF treatment. Error bars represent SEM (n = at least 3 for all conditions); * $P < 0.05$.

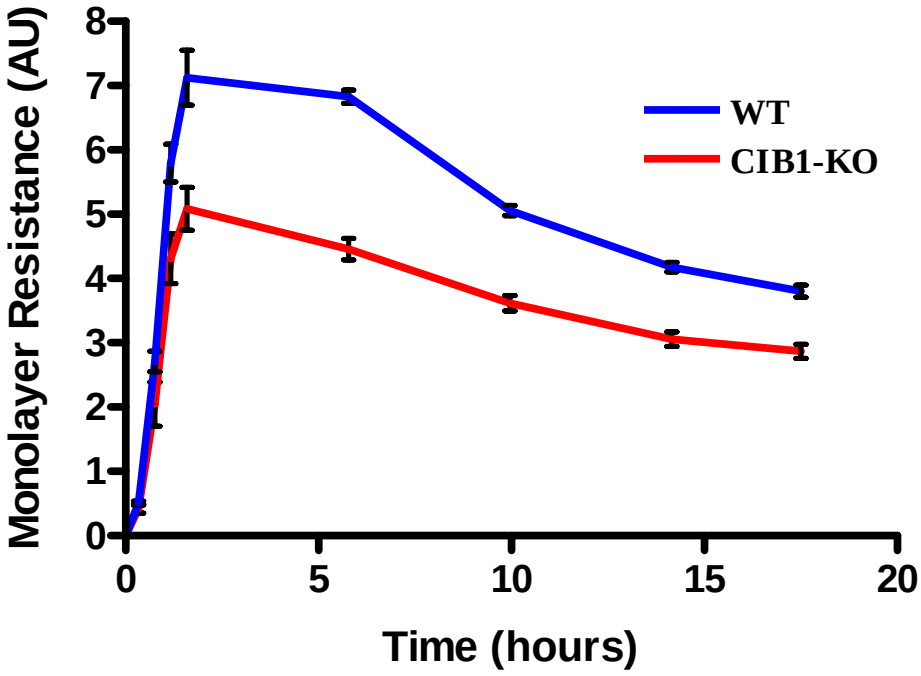

Figure 4-10. Loss of CIB1 decreases EC monolayer ionic resistance. Real-time assessment of ionic resistance across WT and CIB1-KO ECs demonstrates that both cell types adhere to fibronectin in approximately 1.5 hours. CIB1-KO EC monolayers demonstrate decreased ionic resistance upon adhesion and for the following 17 hours thereafter. Assay was performed in replicates of 8, and error bars represent SD.

4.5 Discussion

CIB1 is an important regulatory molecule that is expressed in different tissue types and has various binding partners(34). However, the role of CIB1 in ECs has never been explored. In this study we demonstrate that CIB1 is expressed in ECs and various other vascular structures. We also provide the first evidence to suggest that CIB1 plays an important regulatory role in ECs and can affect various EC functions such as migration, proliferation, nascent tubule formation, and monolayer resistance.

Recent evidence suggests that CIB1 contributes to important intracellular signaling mechanisms. For example, Leisner et al. demonstrated that CIB1 can bind and regulate the activity of PAK1 in different cell types(9). In that study knockdown of CIB1 in REF52 fibroblasts decreased adhesion-induced PAK1 activation at early time points (20 and 45 minutes), but not at later time points (150 minutes). Similarly, here we observe that adhesion-induced PAK1 activation in CIB1-KO ECs is significantly reduced both at 20 and 45 minutes. Thus these findings confirm the dynamic interaction between CIB1 and PAK1, and demonstrate that it likely occurs in different cell types and tissues. Interestingly, at later time points Leisner et al. demonstrated that CIB1-depleted REF52 cells had a rebound PAK1 activation that was dependent on the Rho GTPases Rac and Cdc42(9). However in our study we did not detect in CIB1-KO ECs a rebound activation of PAK1 at later time points (180 minutes), suggesting that this mechanism is either not as profound in ECs or occurs at later time points. These differences could reflect the variation in the pathways used by different cell types or an alteration in pathway usage upon the chronic loss of CIB1 in CIB1-KO ECs.

In our laboratory we previously reported that overexpression of CIB1 in transformed cell lines inhibits migration (unpublished data;(9)). However, in this study we observed that knockdown or deletion of CIB1 from ECs decreased haptotactic migration as well. We also found that reintroduction of CIB1 in CIB1-KO ECs increased migration back to normal levels. This apparent discrepancy can be explained in several ways. First, our studies primarily focused on the role of CIB1 in ECs. It is therefore conceivable that CIB1 has cell type-specific roles that can affect different cellular processes in different ways. Second, previous studies primarily relied on transfection-mediated RNAi knockdown of CIB1. Here we utilize a more efficient method of knockdown (using shRNA) and we also take advantage of CIB1-KO ECs. It is possible that a complete loss of CIB1 alters pathway usage and therefore contributes to the observed discrepancies. Third, PAK1 activation was previously shown to both increase and decrease migration through different signaling pathways. Therefore it is possible that in different settings or in response to different stimuli, CIB1-regulated PAK1 activation can lead to different migratory phenotypes.

Previous reports also show that PAKs can phosphorylate and activate c-Raf(35), MEK1(27), and the MAPKs ERK1/2(36) and p38(25). In this study we demonstrate that CIB1-KO ECs have attenuated adhesion-induced ERK1/2 activation. However, we observe no difference in the activation of other kinases such as p38, PKC, and Akt (data not shown). Furthermore, we observed that the activation pattern of ERK1/2 resembles the activation kinetics of PAK1 (decreased activation only at early time points). This suggests that ERK1/2 activation is a downstream event of CIB1-regulated PAK1 activation. Since efficient

ERK1/2 activation is necessary for various EC functions, it is likely that disrupted ERK1/2 phosphorylation in CIB1-KO ECs is at least in part responsible for the observed decrease in migration, proliferation, and nascent tubule formation upon the loss of CIB1.

Additionally, studies have shown that ERK1/2 activation is necessary for transcriptional up-regulation of MMPs(29;30), which are key modulators of various pathophysiologcial events(37). Constitutively expressed by various types of cells (including ECs), MMP2 degrades ECM and facilitates EC proliferation and migration(31). *In vivo* it also mediates the release of heparin-bound growth factors (such as VEGF and bFGF) from the ECM, and regulates spermatogenesis(38) – a process that also requires CIB1(39). Here we report that CIB1 null ECs express significantly less VEGF and bFGF-induced MMP2. This is a significant observation since MMP2 is detected at high levels in atherosclerotic plaques, aneurysms, and sites of myocardial infarctions(37). A decrease in MMP2 *in vivo* has also been shown to prevent myocardial infarction-induced cardiac rupture(40), as well as tumor progression and angiogenesis(41). Thus these findings suggest that CIB1 may be involved in a wide range of pathologies and cardiovascular disorders *in vivo*.

The decrease in MMP2 expression in CIB1-KO ECs also suggests that perhaps the expression of other important genes is also disregulated upon the loss of CIB1. Indeed, analyses of preliminary global gene expression microarrays suggest that there is a subset of genes that appears to be differentially expressed between WT and CIB1-KO ECs (Appendix A). Most prominently we detect a significant decrease in the expression of the angiopoiten receptors Tie1 and Tie2, which suggests that in addition to VEGF and bFGF, CIB1-KO ECs

may also respond less efficiently to the Ang ligands (Ang1 and Ang2). Furthermore, the expression of EC intracellular junction proteins (such as VE-Cadherin and PECAM-1) is also decreased in CIB1-KO ECs, suggesting that this may be the molecular basis for the observed decreased in CIB1-KO EC monolayer resistance.

It is important to note that loss of CIB1, either by homologous recombination or by depletion via shRNA, did not completely inhibit EC functions. Similarly, loss of CIB1 did not completely abolish *in vitro* PAK1 activation, ERK1/2 phosphorlyation, and MMP2 expression. This suggests that there are likely compensatory mechanisms that facilitate the rescue of EC signaling and function. Although the mechanisms for such compensations are yet to be determined, likely candidates may include the CIB1 homologs CIB2, CIB3, and CIB4. Studies are currently underway to determine whether the expression of any of these homologs is altered upon the loss of CIB1. Another likely candidate for CIB1 compensation may also be calmodulin. This regulatory calcium binding protein is highly homologous to CIB1 and was recently shown to be essential for normal EC function under hypoxic stress(42). Compensatory mechanism may also help explain why CIB1-KO mice develop normally and reach adulthood inconsequentially.

Thus, collectively our data demonstrate that CIB1 is a critical regulator of various EC functions. CIB1 also appears to regulate PAK1 activation and its downstream ERK1/2 signaling pathway in ECs. Further, CIB1-KO ECs have attenuated growth factor-induced function and MMP2 expression. These data suggest that although CIB1-KO mice develop normally, CIB1 may also play an important role in pathological forms of angiogenesis. In

the following chapters we will explore the role of CIB1 in angiogenesis in various different *in vivo* murine angiogenesis models.

4.6 Acknowledgments

We thank David Baltimore and Xiao-Feng Qin for the lentiviral mammalian double-cassette FG-12 vectors; Robert Auerbach and Cam Patterson for MEC and BAEC lines, respectively; Tina Leisner for her assistance with *in vitro* PAK1 kinase assays; James Ferguson for assistance with Northern blotting; Andrew McFadden for modification of some of the lentiviral mammalian FG-12 vectors; Steve Holly of assistance with microscopy; Erika Wittchen and Keith Burridge for their assistance with RT-CES apparatus. This work was supported by grants from the NIH 2-P01-HL45100 (L. V. Parise) and NIH T32-HL-069768 (M. A. Zayed).

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CHAPTER 5

CIB1 is Necessary for Ischemia-Induced Pathological and Adaptive Angiogenesis

5.1 Abstract

In Chapter 4 we demonstrate that CIB1 plays a critical role in EC signaling, function, and MMP2 expression. Since these events are critical for a robust angiogenic response, we tested the role of CIB1 in different forms of angiogenesis *in vivo*. Here we demonstrate that developmental vasculogenesis and angiogenesis are normal in the retinas of CIB1-KO mice. This is not surprising since, besides having male sterility, CIB1-KO mice develop normally and reach adulthood inconsequentially. However, in response to ischemia, CIB1-KO retinas have significantly reduced pathological angiogenesis and retinal neovessel bud formation. Similarly, in a hind-limb ischemia-induced angiogenesis assay, CIB1-KO mice demonstrated reduced hind-paw perfusion, increased hind-paw gangrene and hind-limb muscle tissue damage, and decreased hind-limb adaptive angiogenesis. The observed defects in angiogenesis appears to be specific, since CIB1-KO mice demonstrate no significant difference in other post-ischemic recovery mechanisms such as arteriogenesis. Therefore, these studies provide the first evidence to show that CIB1 has an important role in adult pathological and adaptive forms of ischemia-induced angiogenesis.

5.2 Introduction

Angiogenesis, the process of new blood vessel formation from pre-existing vasculature, occurs in both physiological and pathological situations(1). During embryonic development and after birth, physiological angiogenesis promotes primitive vessel development and permits organ growth(2). In adults, angiogenesis becomes tightly regulated by a balance of pro-angiogenic and anti-angiogenic growth factors and cytokines(1;3). As a result angiogenesis is permitted to take place only in rare physiological situations such as during active wound healing or during tissue regeneration (i.e. in the corpus luteum and the uterine endometrial lining)(1;3-6). However, pathological angiogenesis may also ensue due to imbalances in either angiogenic activators or inhibitors, which can manifest in inconvenient, debilitating, or even life-threatening clinical disorders(1). Several molecular differences between physiological and pathological angiogenesis have so far been identified, suggesting that these two processes are differentially regulated and can arise from different molecular signals(7;8). Identifying exactly which molecules contribute to pathological angiogenesis, but not physiological angiogenesis, will provide clinicians with better therapeutic targets to alleviate angiogenesis-dependent diseases and aid in our understanding of how pathological angiogenesis arises in the first place.

Ischemia-induced angiogenesis is an example of both pathological and adaptive forms of angiogenesis and has a clear association with a subset of common disorders that can affect nearly all types of human tissue(1;9). Among these disorders, age-related macular degeneration (AMD) and diabetic retinopathy are the two most common retinal disorders that

cause vision impairment in adults in the Western world(10;11). Both diseases are believed to be initiated, by among other things, the reduction of oxygen concentrations in the retinal tissue(12). This stimulates the expression and release of growth factors such as VEGF and IGF-1 by surrounding tissue which, in turn, leads to increased vascular permeability and excessive angiogenesis(13;14). Conventional treatment for these disorders has remained the same for the past few decades, and has involved the use of laser photocoagulation-induced destruction of neovasculature(15). Although this therapy is effective in reducing the risk of further vision loss, it does not address the basic biological processes that lead to these diseases and is typically applied after the onset of retinal damage. Thus, identifying and inhibiting molecules that contribute to this pathological induction of angiogenesis may prove to be a more effective therapeutic option.

On the other hand, in some ischemic diseases the desired outcome is to stimulate adaptive angiogenesis in order to facilitate revascularization of ischemic tissue(16). Hence, in diseases like peripheral vascular disease (PVD; also known as peripheral artery occlusive disease) angiogenic therapy has been proposed as an alternative to conventional surgical interventions(17). Such novel therapy would have a large impact since PVD is an extremely debilitating condition that occurs predominantly in elderly populations, and is estimated this year alone to affect nearly 7.4 million individuals – a 100,000 of whom will undergo lower-limb amputation due to limb claudication(9;18). Initial animal studies highlight the promise of this therapy by showing that angiogenic therapy can remarkably increase functional angiogenesis and arteriogenesis (the remodeling and expansion of collaterals between arteries of adjacent vascular beds) following ischemic injury of the limb (reviewed in (19)).

However, so far clinical trials that have focused on the use of non-specific angiogenic growth factors such as VEGF and bFGF, and have had limited success(20;21). It is yet to be determined whether tissue-specific molecules that facilitate ischemia-induced angiogenesis are better candidates for angiogenic therapy.

As demonstrated in Chapter 4, we have found that CIB1 is a critical regulator of ECs. Not only is CIB1 essential for proper activation of PAK1 in ECs, but it also important for various EC functions, including migration, proliferation, tubule formation, and monolayer resistance. To expand upon these findings and to determine whether CIB1 plays a role in angiogenesis *in vivo*, we recently engineered a CIB1-deficient (CIB1-KO) mouse via homologous recombination in embryonic stem cells(22). Although CIB1-KO mice reach all developmental milestones, we hypothesized that CIB1 may still have a role in pathological forms of angiogenesis, including ischemia-induced angiogenesis. Here we demonstrate that although CIB1-KO mice have normal retinal developmental angiogenesis, upon ischemic injury in different tissue they demonstrate increased tissue damage, decreased recovery, reduced plasma VEGF levels, and impaired ischemia-induced angiogenesis. However, arteriogenesis in CIB1-KO mice appears to be unaltered suggesting that CIB1 does not play a critical role in this process. Thus our findings identify CIB1 as a novel and specific regulator of pathological and adaptive forms of ischemia-induced angiogenesis.

5.3 Methods

Retinal isolation. Eyeballs were enucleated from euthanized mice and submerged in 2% PFA, for at least 2 hours, at 4˚C. Eyeballs were washed in PBS and microdissected as previously described. Isolated retinas were permeablized in 70% ethanol and by 0.1% TritonX-100, prior to overnight staining at 4˚C with Alexa Fluor 594 conjugated *Griffonia simplicifolia* isolectin-1-B4 (GSL1-B4; Invitrogen, Carlsbad, CA).

Oxygen-induced retinal angiogenesis. Postnatal day 7 (P7) mice were placed in 75% oxygen for five days (until P12), which induced central avascularity of both CIB1-KO and WT retinas. Following hyperoxic incubation, we housed CIB1-KO and WT neonatal mice for five additional days (until P17) at normoxic conditions, after which mouse eyeballs were enucleated, briefly fixed in 2% PFA, and either micro-dissected to obtain intact retinas or cryopreserved in O.C.T. (IMEB, San Marcos, CA). Isolated retinas were flat-mounted, permeablized, and stained with isolectin GSL-1-B4 (1:100). Fluorescent images of retinas collected with a Nikon inverted microscope were stitched (PhotoFit, TekMate, Anchorage, AK) and the number of neovessel buds (> 7μm) per retina counted (ImageJ; n = 8 mice per group). Interrupted 6μm sections at 50μm intervals were obtained for cryopreserved eyeballs and stained with H&E. Mean capillary area of vessels either directly under or invading the retinal ILM were measured from digitized images obtained from two randomly selected interrupted eyeball sections (n = 4 mice per group).

Unilateral femoral artery ligation. Male and female WT and CIB1-KO mice, at least 15 weeks old, were anesthetized with 1.125% isoflurane supplemented with 2:3 oxygen-air. Rectal temperature was closely maintained at 37.0 ± 0.5˚C. Hair was removed from

hindquarters using depilating cream, with care to avoid erythema. The femoral artery, proximal to the bifurcation of the lateral caudal femoral artery, was aseptically exposed and ligated with 7-0 suture. The wound was irrigated with sterile saline and then the incision was closed.

Laser-Doppler perfusion imaging. Superficial hind-paw plantar and hind-limb ventral adductor thigh regions were monitored with noninvasive measurements obtained using a scanning laser-Doppler perfusion imager (model LD12-IR, Moor Instruments, Wilmington, DE) modified for high resolution and depth of penetration (2mm) with an 830nm-wavelenght infrared 2.5mW laser diode, 100μm beam diameter, and 15kHz bandwidth(23). Prior to measurements, anesthesia, ventilation, and temperature were controlled using the same techniques as with femoral artery ligation. Hind-paw and hind-limb measurements of ischemic and non-ischemic limbs were performed before, and at 1, 7, 14, and 21d after ligation. Hind-paw and hind-limb regions of interest (ROIs) were drawn as previously described(23), to obtain average Doppler velocity in plantar and ventral adductor regions. Average velocity in a ROI was normalized to the area of the ROI, due to unavoidable variations in animal positioning during Doppler scanning. Data were reported as a ratio of ligated-to-nonligated rate of Doppler blood perfusion (n = 11 mice per group).

Hind-paw appearance and hind-limb use score assessments. Appearance and use scores were performed as previously described(24). Appearance score was assessed based on scale; 0 = no change, 1-5 = the number of nails with color change or number of lost nails, and 6-10 = the number of digits lost or digits with color change. Use scores were assessed based

on a scale of; 0 = no change, 1 = no toe flexion, 2 = no hind-paw flexion, 3 = foot or limb dragging. Scores were assessed before femoral artery ligation, and 1, 7, 14, and 21d after ligation (n = 11 mice per group).

Assessment of atrophy, capillary density in gastrocnemius muscle, and X-ray angiography. Twenty-one days after femoral artery ligation, mice were perfused and fixed as previously described with minor modifications(23). Mouse infrarenal abdominal aortas were cannulated and mice were perfused at 100mmHg with phosphate buffered solution (PBS, pH 7.4) containing 3.7mM adenosine and 106μM papaverine. PBS was followed by 4% PFA for at least 20 minutes. Mice were then perfused at 100mmHg for 30m with a barium sulfate (85% w/v; Liquid Barospere, Lafayette Pharmaceuticals) suspension of high viscosity that optimized arterial filling and minimized venous filling. Arterial angiograms were obtained by exposing specimens to an X-ray source (Faxitron MX-20; Faxitron Corporation) for 6.8 seconds at 26kV. As previously described, the number of arterial vessels in WT and CIB1-KO, ligated and non-ligated hind-limbs, was derived from the hind-limb "mid-zone" (n = 5 for each group). Following angiography, ligated and non-ligated hind-limbs were removed from animals and post-fixed in 4% PFA for 48h, with a solution change at 24h. A section of the gastrocnemius muscle was removed *en bloc*, beginning at the Achilles tendon and extending 5mm rostrally. Samples were rinsed in water, placed in 70% ethanol for 48h with shaking and a change of solution at 24h, followed by embedding in paraffin. At least 5 interrupted sections, 50μm apart were obtained and H&E stained. For each gastrocnemius, three 20X images were collected from two randomly selected sections using a Nikon D100 camera attached to a Nikon inverted microscope. Using ImageJ, muscle

atrophy was determined by obtaining the average muscle fiber size within fascicles with a delineated area (n = 5 – 9 mice per group). To assess capillary density, the plasma membrane of capillary ECs in tissue sections were labeled with isolectin GSL-1-B4 (1:100). Using a Nikon TE2000U inverted fluorescent microscope with an OrcaER, three 20X images were collected for two randomly selected sections. Within delineated areas of muscle fascicles (quantified by ImageJ software), capillaries were identified as GSL-1-B4-positive vessels with diameter < 7μm. Capillary density was reported as the ratio of capillary-to-muscle fiber number (n = 5 – 9 mice per group).

ELISA. Quantitative sandwich ELISA was performed according to manufacturer's instructions (R&D Systems) to detect VEGF in mouse plasma and tissue homogenates (n = 8-11 per group).

Animal regulations. All housing, breeding, and experimental procedures performed with mice were in accordance with national guidelines and regulations, and were approved by the UNC-CH Institutional Animal Care and Use Committee (IACUC).

Statistical analysis. We compared continuous variables with either the Student's t-test or the non-parameteric Mann-Whitney U test. We considered $P < 0.05$ to be significant. For animal studies, male and female mice demonstrated no differences; thus they were combined. Statistical significance was determined by unpaired t-test for comparisons across animal groups.

5.4 Results

5.4.1 Vascular development in CIB1-KO mouse retinas is normal.

Mice lacking CIB1 develop and reach adult reproductive age normally(25). Despite this, an underlying defect in developmental angiogenesis may still exist *in vivo* in CIB1-KO mice, yet not manifest overtly. The retina is one of the most highly vascularized tissues in an adult mouse, but is avascular at birth(26). With physiological hypoxia and increased metabolic demand during the first week of development in the mouse, the retinal vasculature begins to emerge from the optic nerve on postnatal day 0 (P0) and its development is complete by P18(27). To study whether developmental angiogenesis is at all affected in CIB1-KO mice, we assessed the extent of vascularization in isolectin GSL1-B4 stained WT and CIB1-KO retinal flatmounts.

At 2 days post-partum (P2) WT and CIB1-KO had similar hyaloid vascular systems (Figure 5-1A). These large and highly interconnected vessels emerge from the optic nerve prior to birth and supply the retina with nutrients and gases via diffusion. At P4, the hyaloid vascular system is also observed in both WT and CIB1-KO mice, and appears to cover the entire surface of the retina. However, at P4, the retinal vasculature starts to also emerge and extends in a radial fashion toward the periphery of the retina (Figure 5-1B). These newly vascularized areas are approximately the same size in both WT and CIB1-KO retinas. By P8, the retinal vasculature has nearly reached the periphery of WT and CIB1-KO retinas and

forms many interconnections to generate a two-dimensional vascular system (Figure 5-1C). At this point the hyaloid vasculature has also started to regress. By P12, both WT and CIB1-KO retinas are fully vascularized, and at this time the vasculature is multi-dimensional and complex (Figure 5-1D). Evidence of vascular pruning can also be observed in WT and CIB1-KO retinas at this stage, with reorganization of retinal capillaries away from adjacent newly formed arterioles (Figure 5-1E). Similarly, adult retinas in both groups are also nearly identical (Figure 5-1F). Using laser-scanning confocal microscopy, all three retinal vascular layers are observed in both WT and CIB1-KO adult retinas (Figure 5-1G). Thus these findings confirm that there is no defect in the development of the retinal vascular in CIB1-KO mice.

5.4.2 CIB1 deficiency leads to decreased oxygen-induced retinal angiogenesis.

It has been repeatedly demonstrated that EC regulatory molecules that do not contribute to developmental angiogenesis, may still have a significant role in pathological and adaptive forms of angiogenesis(7;8). We previously demonstrated that CIB1-KO ECs have disrupted signaling and angiogenic function. Therefore we hypothesized that although CIB1-KO retinal vascular developmental appears normal, CIB1-KO mice may still have a significant defect in pathological retinal angiogenesis. To study this we performed an oxygen-induced retinal angiogenesis assay that models the ischemic disease of retinopathy of prematurity(28). In this assay, P7 WT and CIB1-KO littermate neonatal mice are exposed to elevated inspired oxygen concentrations. This induces obliteration of the central retinal

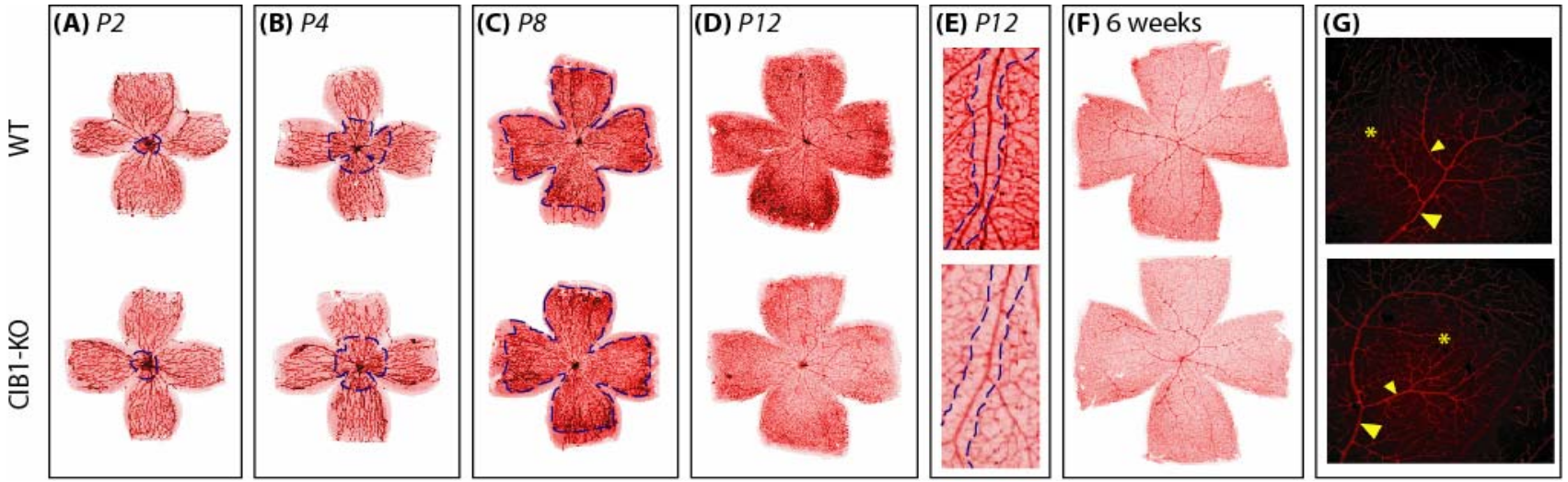

Figure 5-1. Retinal vascular development is normal in CIB1-KO retinas. (**A**) WT and CIB1-KO P2 retinas. Interconnected hyaloid vasculature can be seen superimposed upon avascular retinas. Emerging retinal vasculature can be seen at the center of the retina at the optic cup (outlined in blue). (**B**) P4 retinas still demonstrate superimposed hyaloid vasculature and both WT and CIB1-KO retinas show expanding retinal vasculature. (**C**) Hyaloid vasculature has regressed in P8 WT and CIB1-KO retina and they are both nearly fully vascularized. (**D**) Vessels reach the perimeter of WT and CIB1-KO P12 retinas, and normal vascular pruning around established retinal arterioles (**E**). (**F**) Adult, 6 week old WT and CIB1-KO retinas are fully vascularized and confocal microscopy shows the formation of all three retinal layers; superficial, indicated by large arrowhead; intermediate, indicated by small arrowhead; and deep, indicated by *.

capillaries, which then leads to hypoxia in the mouse neonatal retina. Subsequent return of P12 neonates to normoxia induces exuberant angiogenesis, resulting in the formation of aberrant capillary neovessel buds that can be visualized in isolectin GSL1-B4 stained retinal flat-mounts. Although examination of P17 WT and CIB1-KO retinas demonstrated no difference in vascularized versus non-vascularized retinal areas (visualized in Figure 5-2A), a significant difference was observed in the number of retinal neovessel buds that formed in each group. CIB1-KO retinal flat-mounts demonstrated 22% fewer neovessel buds that were less clearly defined compared to WT littermate controls (Figure 5-2A, and quantified in 5-2C). Since these buds are composed of clusters of tortuous vessels that protrude through the retinal inner limiting membrane (ILM)(29), retinal cross-sections were also obtained to assess the size and morphology of these neovessels. WT retinal cross-sections displayed large protruding microvessels that occasionally ruptured through the ILM, whereas CIB1-KO retinal cross-sections showed blood vessels directly under the ILM that were smaller in size and did not protrude through as frequently (Figure 5-2D; and quantified in Figure 5-2E). In CIB1-KO retinas, the ILM was also almost always intact (Figure 5-2E). Thus, these data provide the first evidence that demonstrates that CIB1-KO mice have impaired pathological neovascularization in response to ischemia.

5.4.3 CIB1 deficiency delays post-ischemia hind-limb re-perfusion and recovery

A common mouse model for peripheral vascular disease is experimentally-induced hind-limb ischemia through unilateral femoral artery ligation(30). In this model, blood flow

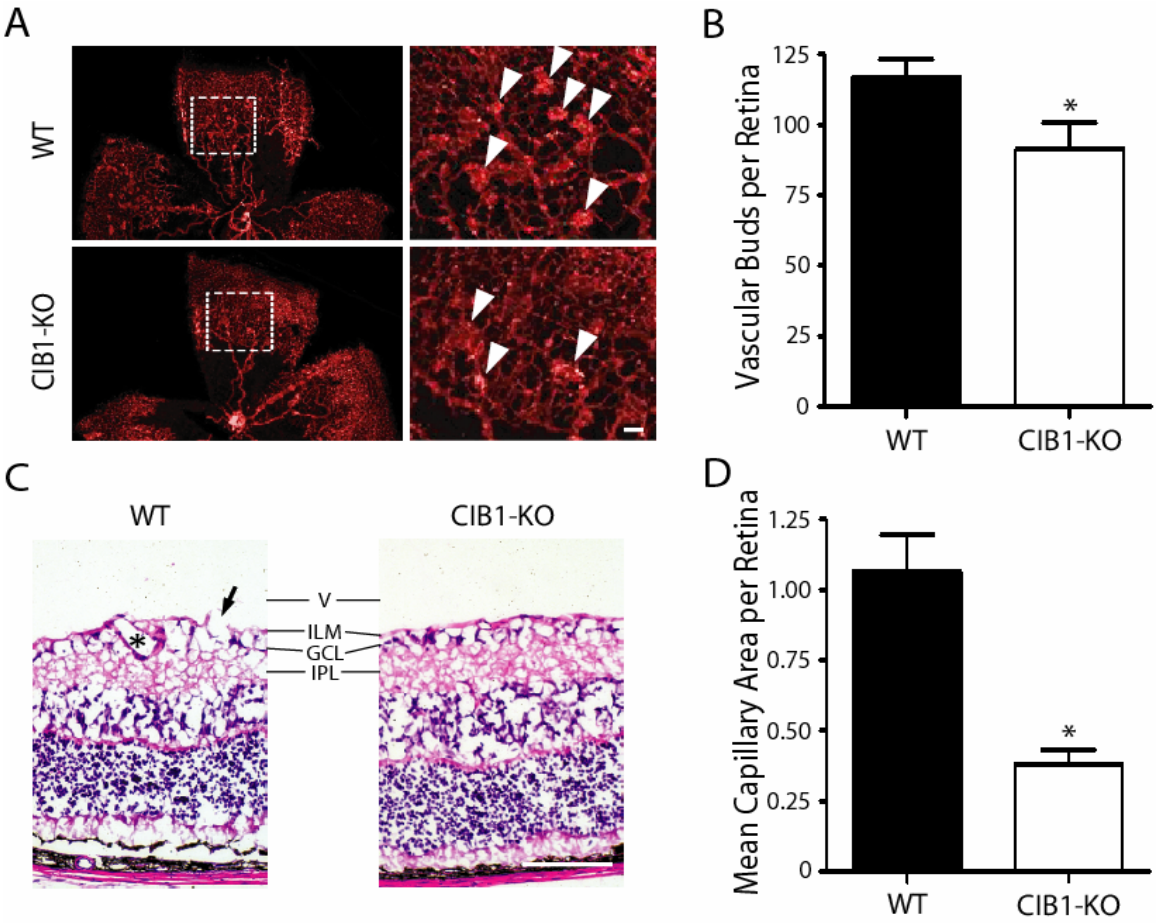

Figure 5-2. Oxygen-induced retinal neovascularization is decreased in CIB1-KO mice. (**A**) Isolectin GSL1-B4 stained flat-mounted retinas from WT and CIB1-KO neonatal mice that have undergone oxygen-induced retinal angiogenesis (left panel). Vascular buds that formed in WT and CIB1-KO ischemic retinas are indicated by arrowheads in magnified retinal areas (right panel). Scale bar, 25µm. (**B**) Quantification of vascular buds per retina in WT and CIB1-KO mice ($n = 8$ per mouse genotype). (**C**) Representative H&E stained cross-sections from WT and CIB1-KO retinas after exposure to hyperoxia (75% oxygen) followed by return to room air. The WT retina shows microvessel protrusion (*) and rupture (arrow) through the ILM. Scale bar, 50µm. (**D**) Quantification of mean capillary area of microvessels directly under the ILM in WT and CIB1-KO retinas following ischemic injury ($n = 4$ mice for each mouse genotype). V, vitreous; ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer. Error bars represent SEM; * $P < 0.05$.

to the hind-limb is effectively eliminated, which rapidly induces ischemia and post-ischemic recovery mechanisms (i.e. angiogenesis and arteriogenesis). To extend our observations from the oxygen-induced retinal angiogenesis assay, we performed this procedure to assess blood flow and tissue recovery in the hind-paw regions (plantar regions of the hind-limbs) of WT and CIB1-KO littermate mice.

Using high-resolution, non-invasive, laser scanning Doppler velocimetry(23), we determined that following ligation, blood re-perfusion in the hind-paws of WT mice was nearly always greater than CIB1-KO littermates (Figure 5-3A). By 14 days after femoral artery ligation, 90% of the perfusion was restored in WT mice but only 60% in CIB1-KO mice (Figure 5-3B). Furthermore, ischemia-induced tissue damage was more severe in CIB1 null mice. Hind-paw toe regions of CIB1 null mice consistently presented with lower perfusion levels due to the onset of gangrene (Figure 5-3A).

Incidence of nail and toe discoloration, toe loss, hind-paw cyanosis and edema, as well as impaired hind-limb function was assessed using previously described appearance and use scores(24). At 14 days after femoral artery ligation, CIB1-KO mice presented with a nearly 3-fold increase in appearance score compared to WT littermates (Figure 5-4A). Similarly, use scores for CIB1-KO mice were elevated, indicating that CIB1-KO mice experienced significantly reduced hind-paw toe and hind-limb flexion (Figure 5-4B). At 21 days after liagation, WT mice regained full function of the ischemic hind-limb, but CIB1 null mouse hind-limbs never fully regained function, suggesting that muscle tissue damage in these mice was more severe.

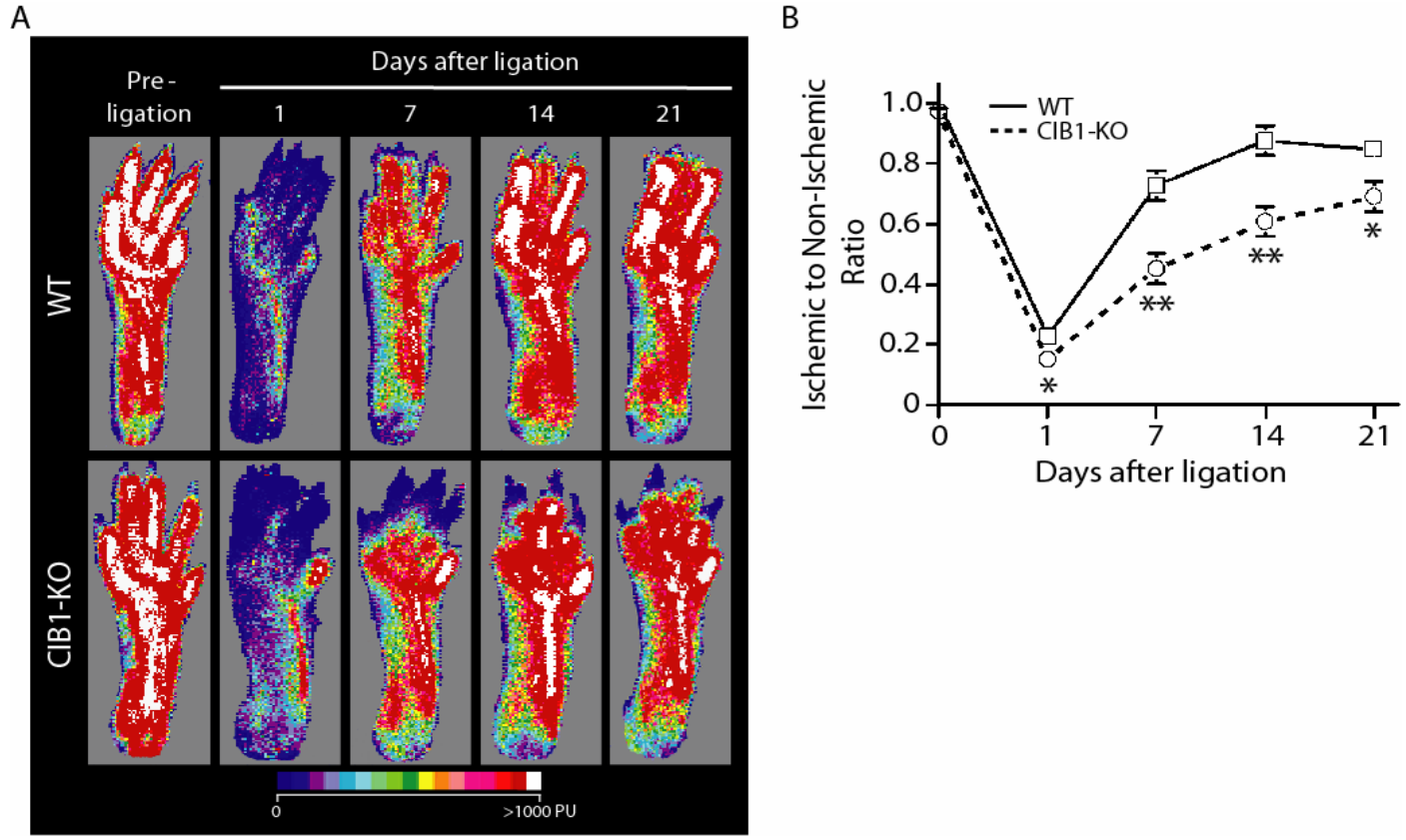

Figure 5-3. Reduced perfusion and recovery in CIB1-KO mouse ischemic hind-paws following femoral artery ligation. (**A**) Scanning laser-Doppler perfusion images of ventral hind-paw surfaces of ischemic hind-limbs of WT and CIB1-KO mice. Relative flow velocity is indicated by 6-hue pseudocolor, where gray represents zero and white represents maximal velocity (range of velocity values = 0–5000 PU, arbitrary units). Pre-ligation relative blood flow velocity is high in both groups, as indicated by white and red. One day after ligation, blood flow is largely eliminated to the hind-paw as indicated by blue. Over time CIB1-KO mice show evidence of toe gangrene in the ischemic hind-paw. (**B**) Quantification of perfusion at 7, 14, and 21d post-femoral artery ligation in WT and CIB1-KO mice (n = 11 per mouse genotype; * $P < 0.05$, ** $P < 0.001$).

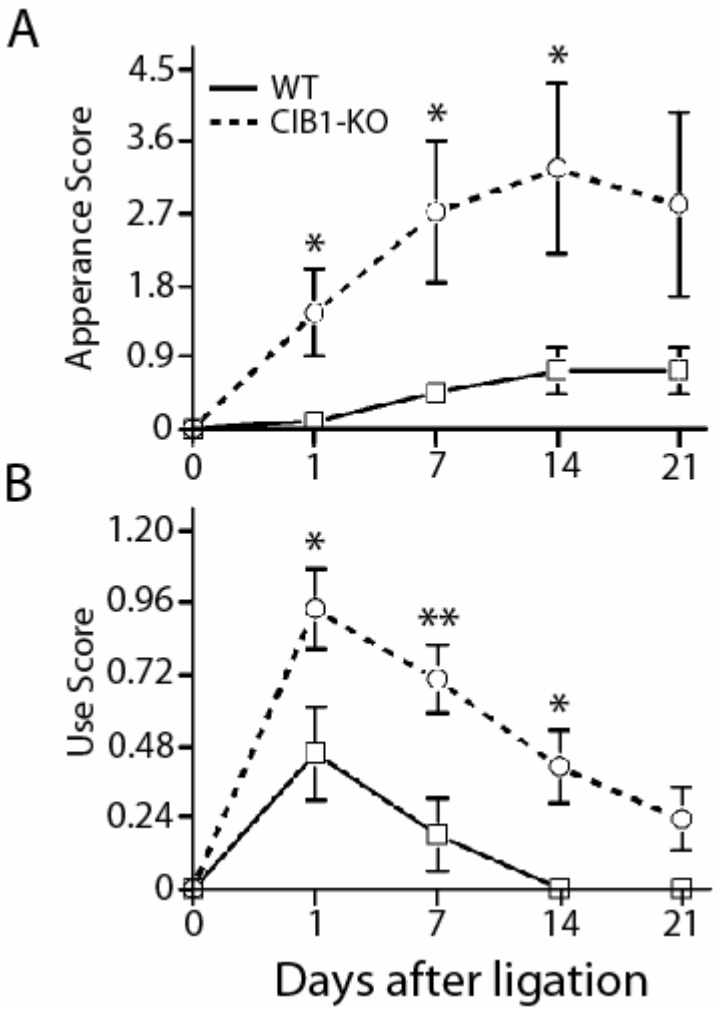

Figure 5-4. Reduced recovery in ischemic CIB1-KO hind-paws and hind-limbs. (**A**) As previously described, and as explained in the methods section, hind-paw and hind-limb appearance and use scores were obtained to assess the rate of recovery following ischemia in WT and CIB1-KO mice. (n = 11 per mouse genotype; Mann-Whitney U test, * $P < 0.05$, ** $P < 0.01$). Error bars represent ± SEM.

Of the muscles in the distal hind-limb, the gastrocnemius muscle experiences the most significant amount of ischemia upon femoral artery ligation(31;32). Decreased hind-limb flexion, as demonstrated by elevated hind-limb use scores in CIB1-KO mice (Figure 5-4B), suggests that these mice have increased gastrocnemius muscle tissue atrophy. To determine the extent of this in WT and CIB1-KO mice, muscle tissue sections were obtained at 21 days from both ischemic and non-ischemic gastrocnemius muscles. As previously reported, no significant difference in muscle fiber size or appearance was observed in WT ischemic and non-ischemic gastrocnemius muscles (Figure 5-5A). This demonstrates that at 21 days after femoral artery ligation WT mice are able to recover normally. However, significant atrophy and fibrosis were detected in the gastrocnemius of CIB1-KO ischemic hind-limbs. Average muscle fiber size in CIB1-KO ischemic gastrocnemius muscles was reduced by 23%, but in WT mice by only 6% (Figure 5-5B). Thus, these data show that CIB1-KO mice experience more profound tissue damage following ischemic insult.

5.4.4 CIB1-KO mice have decreased hind-limb ischemia-induced neovascularization.

A deficiency in either or both angiogenesis or arteriogenesis (the remodeling of pre-existing arteriole-to-arteriole collateral vessels) could explain the difference in tissue damage observed in WT and CIB1-KO mice following femoral artery ligation(33). To first test the contributions of angiogenesis, we determined capillary densities in gastrocnemius muscles, which experience substantial ischemia immediately after femoral artery ligation(23). Compared to control, there was less of an increase in capillary density in CIB1-KO ischemic

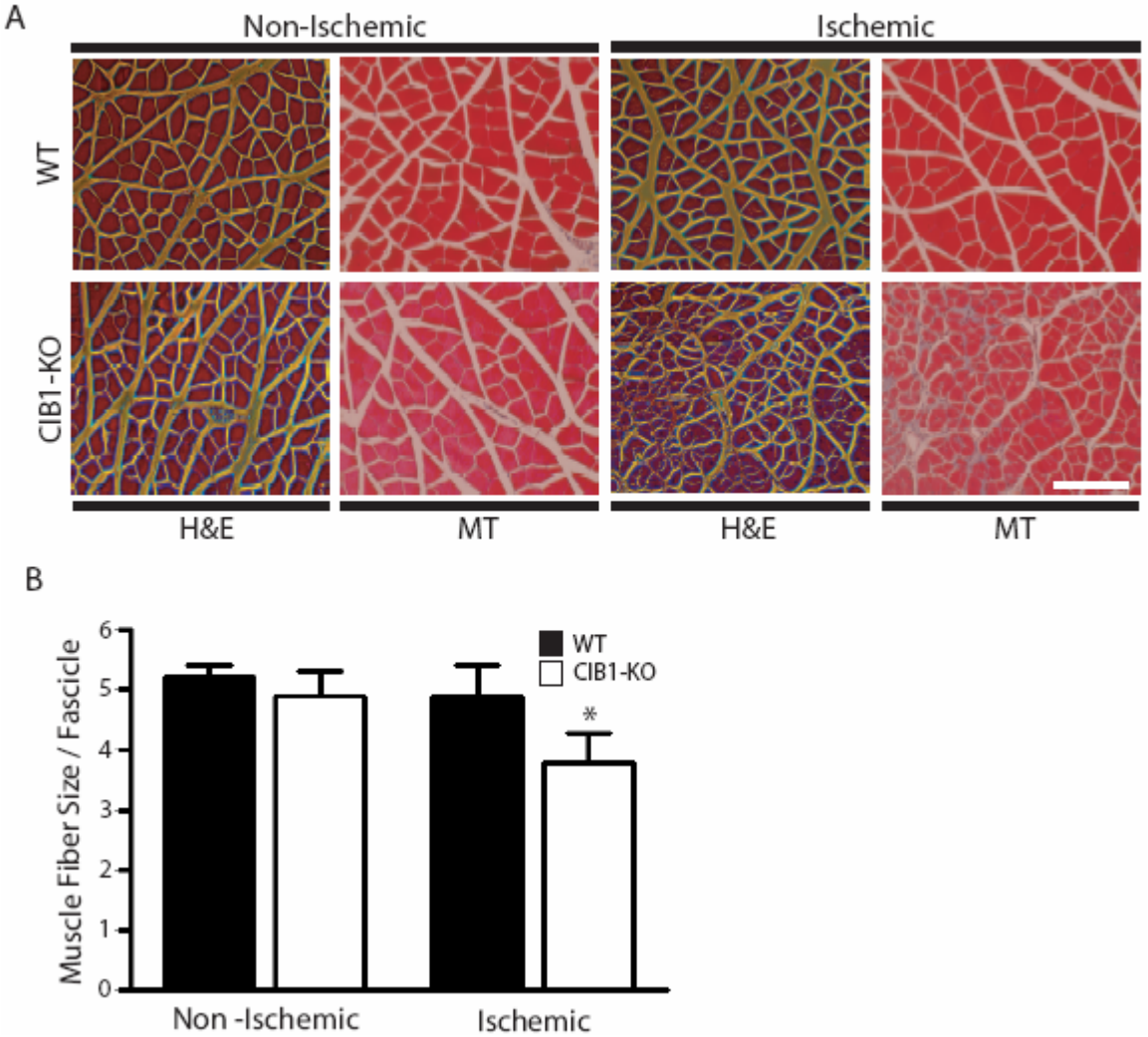

Figure 5-5. Decreased recovery in CIB1-KO mouse ischemic gastrocnemius muscles. (**A**) Contrast enhanced H&E, and Masson's trichrome (MT)-stained sections of WT and CIB1-KO mouse, ischemic and non-ischemic, hind-limb gastrocnemius muscles. Scale bars, 50μm. (**B**) Mean muscle fiber size (μm^2) per muscle fascicle in ischemic and non-ischemic, WT and CIB1-KO gastrocnemius muscles. Error bars represent SEM (n = 5-9 per mouse genotype; * $P < 0.05$).

tissue, as determined by isolectin GSL1-B4 staining (Figure 5-6A). However, since the reduced muscle fiber size in CIB1-KO mice can confound this interpretation, capillary number-to-muscle fiber number ratio was determined(23). The ratio was significantly elevated in WT ischemic gastrocnemi, yet virtually unchanged for CIB1-KO mice (Figure 5-6B), thus providing evidence that CIB1-KO mice have reduced ischemia-induced adaptive angiogenesis. This decreased angiogenic response likely contributes to the increase in muscle tissue damage and delayed recovery in ischemic CIB1-KO hind-limbs.

The decreased angiogenesis in CIB1-KO mice following hind-limb ischemia could be due to either defects in CIB1-KO EC function and signaling (Chapter 3), or decreased availability of angiogenic growth factors from surrounding tissue. Indeed, less VEGF was detected in the plasma of CIB1-KO mice 5 days after femoral artery ligation (Figure 5-7A). Initial assessments of CIB1-KO plasma using Luminex technology suggested that the difference in VEGF plasma levels was specific, since levels of other plasma proteins (i.e. fibrinogen, CD40 ligand, and haptoglobin) were not significantly affected (data not shown). Using ELISA, we also did not detect a difference in VEGF expression between ischemic and non-ischemic gastrocnemi of WT or CIB1-KO mice (Figure 5-7B). Therefore, the decreased angiogenic response is likely due to a combination of reduced function and signaling of CIB1-KO ECs and perhaps low VEGF levels in plasma, but unlikely to be due to low VEGF levels in the gastrocnemi.

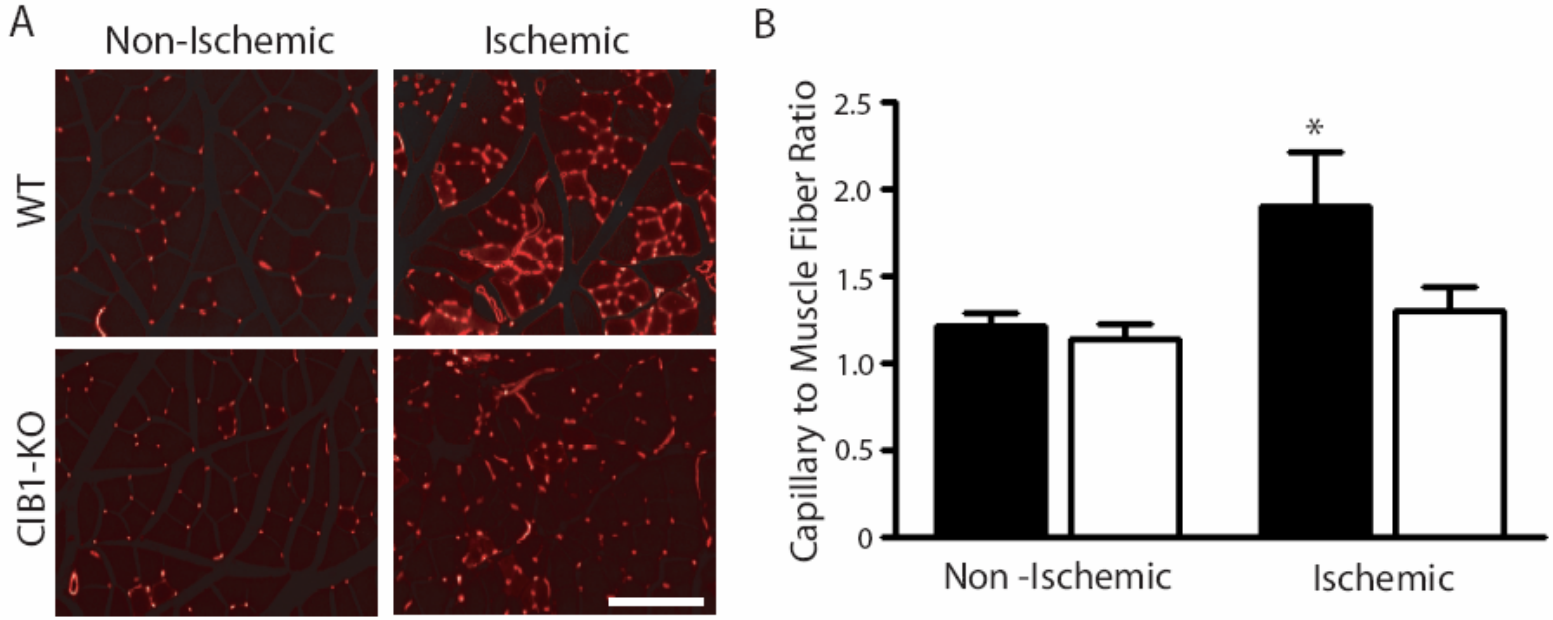

Figure 5-6. Decreased angiogenesis in CIB1-KO mouse ischemic gastrocnemius muscles. (**A**) Isolectin GSL1-B4 staining pattern of capillary EC in WT and CIB1-KO, ischemic and non-ischemic, gastrocnemius muscles. Scale bars, 50μm. (**B**) Quantification of microvessel density, expressed as ratio of capillary number-to-muscle fiber number per fascicle, in WT and CIB1-KO mice. Error bars represent SEM (n = 5-9 per mouse genotype; * $P < 0.05$).

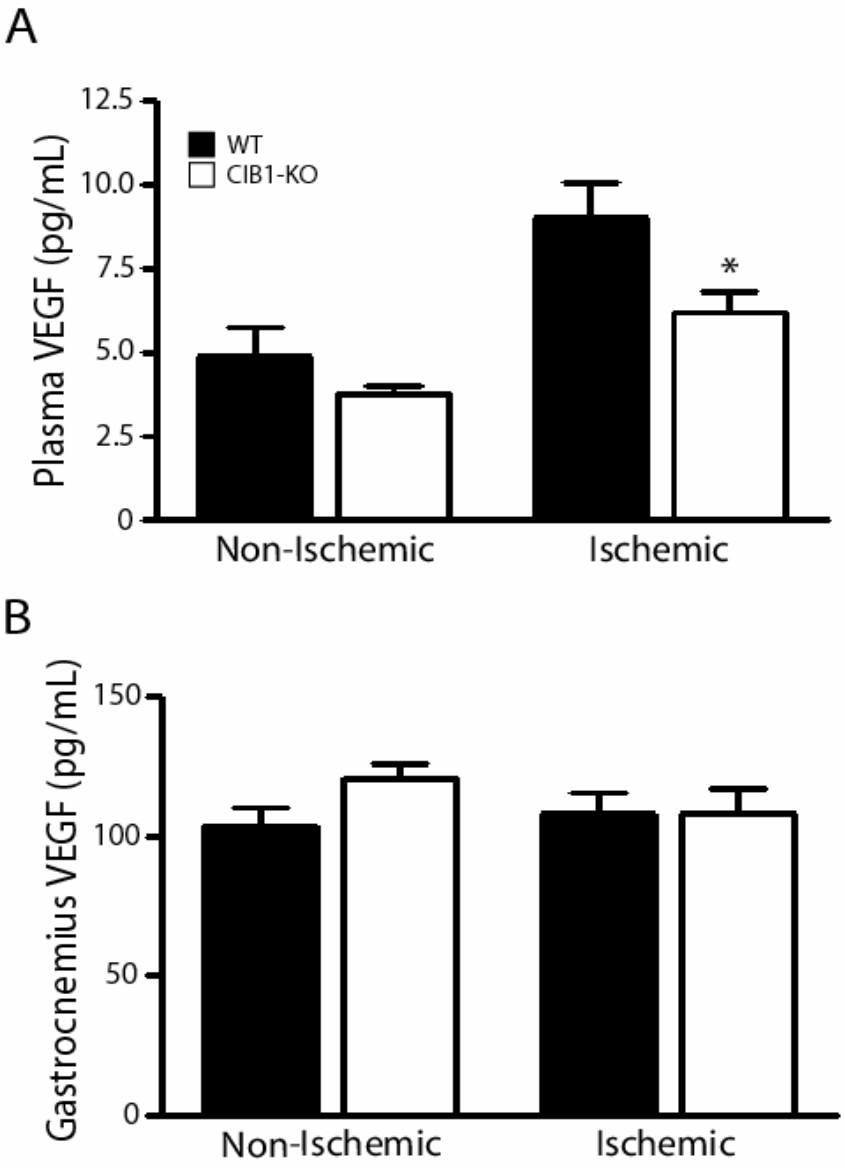

Figure 5-7. Decrease in VEGF plasma levels in CIB1-KO mice but not in gastrocnemius muscles. (**A**) VEGF levels in EDTA plasma pre- and 5 days post-femoral artery ligation in WT and CIB1-KO mice, as determined by ELISA. Error bars represent SEM (n = 4-10 mice per condition; * $P < 0.05$). (**B**) No difference was observed in VEGF levels in gastrocnemius muscle tissue homogenates of WT and CIB1-KO mice (n = 8-10 mice per condition).

5.4.5 Ischemia-induced arteriogenesis in CIB1-KO mice is unaltered.

Impaired arteriogenesis (expansion of collateral diameter) may also contribute to reduced recovery and perfusion in CIB1-KO ischemic hind-limbs, since it is initiated by flow-induced shear stress in pre-existing collaterals following arterial ligation(34). To test this we used laser-Doppler velocimetry to measure perfusion in the middle of the hind-limb ventral adductor region, which contains the major collaterals capable of restoring blood flow to arterial trees below the point of the femoral artery ligation. Following femoral artery ligation, increased perfusion was observed in these regions in both WT and CIB1-KO ischemic hind-limbs (Figure 5-8A; quantification in Figure 5-8B). Consistent with other reports, perfusion in WT mice gradually increased and peaked at 7 days, followed by resolution by day 21(23). However, in CIB1-KO mice an increase in perfusion in the collateral zone rose more slowly (Figure 5-8B). This could reflect the decreased angiogenesis in the ischemic distal limb (Figure 5-6A & 5-6B), rather than impaired arteriogenesis. This interpretation is supported by X-ray angiographic analysis 21 days post-ligation, which indicated no significant difference in the number of vessels detected in the collateral zone (Figure 5-9A; and quantified in Figure 5-9B). Angiography also revealed no difference in the number of vessels detected at baseline in the non-ligated WT and CIB1-KO hind-limbs (data not shown). Thus, although the perfusion patterns are different for WT and CIB1-KO mice, positive collateral remodeling and formation appears unaltered.

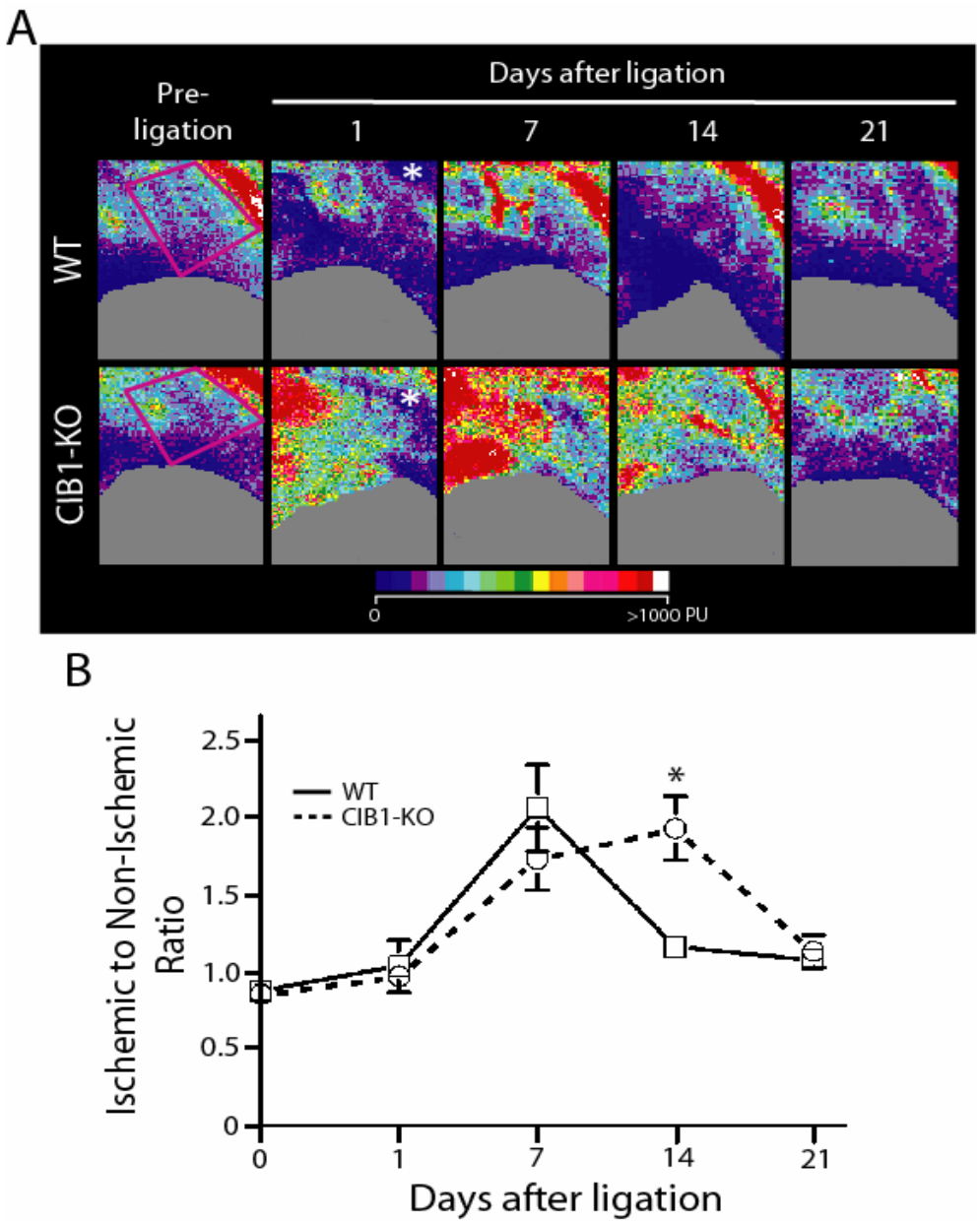

Figure 5-8. Hind-limb ischemia-induced collateral perfusion increased less rapidly in CIB1-KO mice. (**A**) Scanning laser-Doppler perfusion images of the ventral surface of upper hind-limbs from the same mouse show a gradual increase then decrease in perfusion over time. Wound suture line is identified by (*). (**B**) Quantification of perfusion was performed as previously described, using areas marked in magenta (**A**), and expressed as the ratio of perfusion in ischemic-to-non-ischemic hind-limb ventral adductor surfaces of WT and CIB1-KO mice. Perfusion in WT mice peaked at 7d post-ligation, but in CIB1-KO mice it rose more slowly and peaked at 14d. Values are means ± SEM (n = 11 for each mouse genotype; * $P < 0.05$).

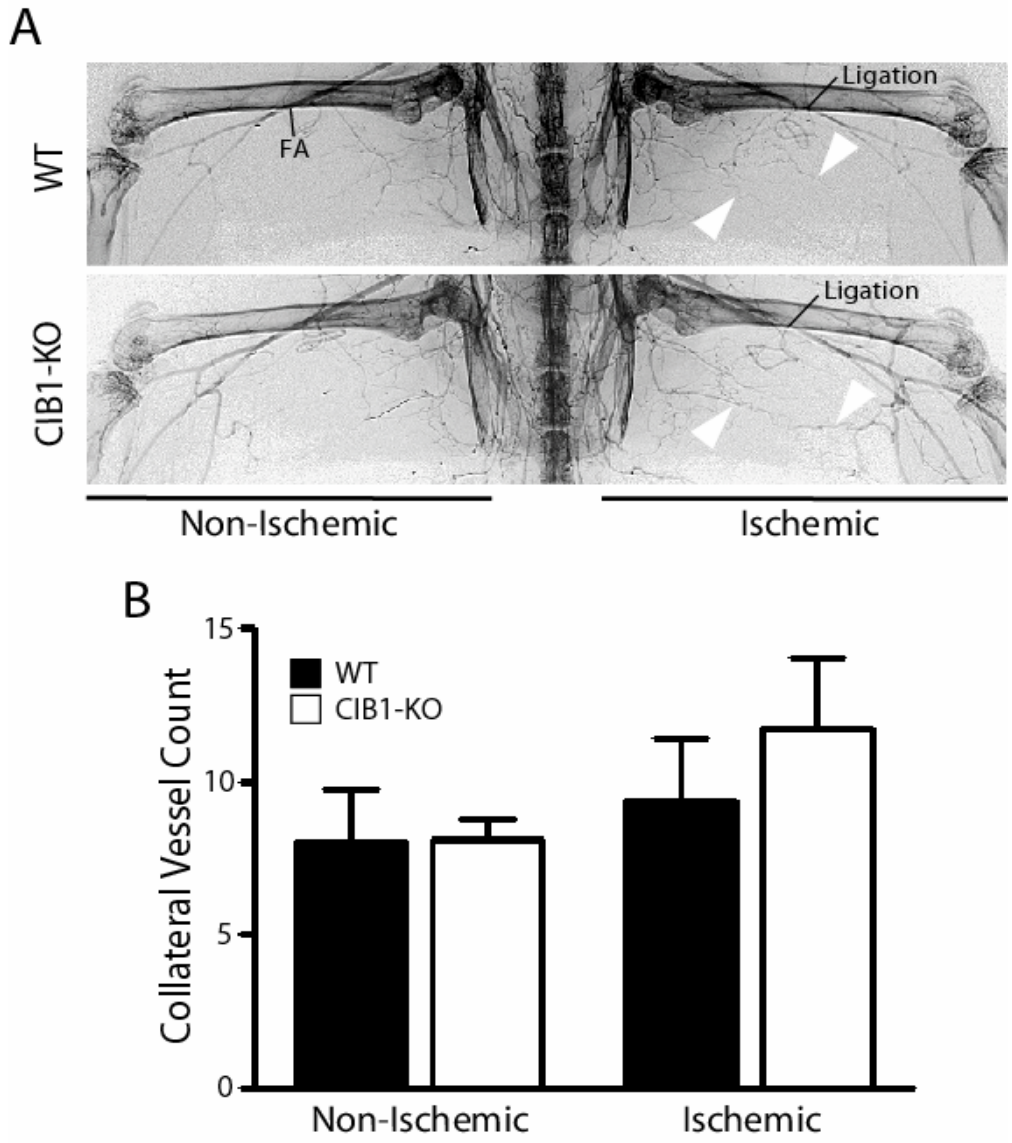

Figure 5-9. Hind-limb ischemia-induced arteriogenesis is unaltered in CIB1-KO mice. (**A**) Postmortem X-ray angiography of perforating collateral arteries in the adductor region 21d post-ligation. Point of femoral artery (FA) ligation shows a disruption in the course of the femoral artery. Examples of newly formed collaterals that connect the femoral artery to distal arteries are identified with arrowheads. (**B**) Average number of newly formed collaterals in the adductor "mid-zone" is not significantly different between WT and CIB1-KO ischemic and non-ischemic hind-limbs. Error bars represent SEM (n = 5-7 per mouse genotype).

5.5 Discussion

Mice deficient in CIB1 grow and develop normally, reach reproductive age, and have no overt vascular phenotype. So far the only defect identified in CIB1-KO mice is male sterility due to a disruption of the haploid phase of spermatogenesis(22). In Chapter 4 we show that CIB1-depleted ECs have attenuated signaling and decreased function. Furthermore, preliminary gene array studies in CIB1-KO ECs reveal decreased expression of genes that are critical for vasculogenesis and angiogenesis (i.e. Notch 1 and Tie2; Appendix A). Not surprisingly however, we observe here that vasculogenesis, which facilitates blood growth in retinas of neonatal mice between P0 and P10, progresses normally in CIB1-KO mice. This confirms that CIB1 does not affect the rate of vasculogenesis and is not essential for this process. Furthermore, we do not observe a significant difference in the development of intermediate and deep capillary beds that form solely by angiogenesis between P8 and P18. Thus, in lieu of our previous findings (described in Chapter 4), these observations suggest that compensatory mechanisms are likely taking place during developmental vasculogenesis and angiogenesis to override the loss of CIB1 and facilitate normal vessel growth. Likely candidates for such compensation were previously mentioned in Chapter 4, and include the CIB1 homologs CIB2, CIB3, CIB4, and calmodulin.

Although CIB1-KO mice have no defect in developmental angiogenesis, we observed in two different murine angiogenesis models (the oxygen-induced retinal angiogenesis assay and the hind-limb ischemia-induced angiogenesis assay) that CIB1-KO mice have impaired ischemia-induced pathological and adaptive angiogenesis. Similarly, previous reports also

demonstrated that genes not essential for embryonic vasculogenesis and developmental angiogenesis, may still have significant roles in pathological and adaptive forms of angiogenesis (see Chapter 2, Table 2-3). Prominent examples of this include KO mice for bFGF and the VEGF homolog P1GF(8;35). Like CIB1-KO mice, embryonic angiogenesis and development is normal in both bFGF-KO and P1GF-KO mice. However, deletion of bFGF or P1GF also leads to significantly impaired pathological angiogenesis *in vivo* during ischemia, inflammation, wound healing, and tumor growth (discussed in (7)). Other examples of impaired ischemia-induced angiogenesis can also be observed in KO mice deficient in the intracellular non-receptor tyrosine kinase Etk (endothelial/epithelial tyrosine kinase)(7), MMP9(36), caveolin-1(37), ICAM-2(38), the prostaglandin E2 receptor(39), thrombopoietin (TPO)(40), and adiponectin(41). Interestingly, some KO mice also demonstrate enhanced ischemia-induced angiogenesis despite having unaltered developmental angiogenesis, as is the case for integrin β3- and β5-KO mice(42). Our study here indicates that CIB1 is another member of this growing group of genes that contribute to pathological and adaptive forms of angiogenesis but not to physiological forms of angiogenesis.

In vivo, ischemia has several components, such as stasis of blood flow, decreased (acidic) pH, reduced glucose availability, accumulation of waste products (i.e. metabolites and lactic acid)(9). However the primary feature of ischemia that affects vascular tissue is hypoxia. This is demonstrated *in vitro* where induction of hypoxia alone in vascular cell cultures (i.e. cultures of ECs and vascular smooth muscle cells) can significantly alter gene expression and cellular behavior(43). One key regulator of the hypoxic response is the

heterodimeric transcription factor HIF-1(44). Under hyopoxic conditions HIF-1 induces the expression of EC mitogens such as VEGF(45). In turn, secretion of VEGF activates ECs to facilitate further growth factor release (paracrine secretion of bFGF and PDGF), increase permeability, and induce further angiogenesis. We show here that following hind-limb ischemia, VEGF plasma levels are reduced in CIB1-KO mice. Pervious reports have demonstrated a direct-correlation between systemic VEGF plasma levels and the level of ischemia-induced angiogenesis(46). Thus, decreased VEGF plasma levels in CIB1-KO mice may be one contributing factor to the observed decrease in angiogenesis in the ischemic gastrocnemius mucles of CIB1-KO mice.

In addition, a robust angiogenic response following ischemic injury is highly dependent on EC signaling and function. EC MMP2 expression is known to contribute to various forms of pathological angiogenesis, including ischemia-induced retinal angiogenesis(47). MMP2 contributes to angiogenesis by degrading ECM proteins (i.e. collagen, fibronectin, and laminin) and proteolytically activating other proMMPs, such as proMMP9, which is also essential for mediating ischemia-induced angiogenesis(48). Accordingly, genetic deletion of MMP2 or MMP9 significantly reduces postnatal ischemia-induced angiogenesis(36;49). In Chapter 4, we demonstrated that CIB1-KO ECs have significantly attenuated adhesion-induced signaling and decreased growth factor-induced migration, proliferation, tubule formation, and MMP2 expression. Thus it is very likely that these defects in CIB1-KO ECs contribute to a large extent to the blunted angiogenic response observed in both CIB1-KO ischemic retinas and hind-limb gastrocnemius muscles.

But CIB1-KO mice do not have a defect in arteriogenesis, which has also been show to involve EC function and MMP2-induced ECM degradation(33). The differences in CIB1-mediated angiogenesis and arteriogenesis likely arise from the different mechanisms that induce each process. Previous reports demonstrate that ischemia-induced adaptive angiogenesis in the gastrocnemius is primarily driven by hypoxia in the surrounding tissue(50;51). In contrast, arteriogenesis occurs independent of hypoxia (often at sites proximal to the hypoxic zone), and is instead induced by physical forces such as fluid shear stress(52). These physical forces arise from a rapid change in blood pressure in a vascular bed following arterial occlusion and they force pre-existing collaterals to remodel in order to accommodate ischemic tissue demands. X-ray angiography demonstrated no significant difference in neo-collateral formation in the adductor regions of CIB1-KO and WT hind-limbs, therefore suggesting that this post-ischemic recovery mechanism is unaltered in CIB1-KO mice.

However, emerging reports argue that arteriogenesis is also an active growth process, rather than just a simple passive dilatation process caused by altered blood pressure(33). *In vitro* evidence suggests that fluid shear stress can also activate ECs and alter their cytoskeletal dynamics(32). It is also hypothesized that *in vivo* neo-collateral ECs can become activated and undergo increased proliferation and migration, thus ultimately facilitating an increase in collateral vessel diameter. Bone marrow-derived macrophages, endothelial progenitor cells, and hemangiocytes can also contribute to the process of arteriogenesis(40). Thus arteriogenesis obviously involves more than just one process and is regulated by multiple mechanisms that work in concert. Hence, redundancies in these

mechanisms may help explain why CIB1-KO mice have unaltered arteriogenesis, eventhough CIB1-KO ECs demonstrate reduced function (Chapter 4).

Therefore, collectively our study identifies a previously uncharacterized role for CIB1 in the vasculature, and for the first time demonstrates that it has a role in angiogenesis *in vivo*. Our study also demonstrates that CIB1 is critical for both pathological forms of ischemia-induced angiogenesis (in the retina) as well as adaptive forms of angiogenesis (in the hind-limb). Finally, we provide evidence to show that CIB1 does not contribute to ischemia-induced arteriogenesis. These are important findings since they suggest that CIB1 can be a novel and specific therapeutic target for pathological angiogenesis *in vivo*.

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CHAPTER 6

Role of CIB1 in Growth Factor-Induced and Tumor-Induced Pathological Angiogenesis

6.1 Abstract

In previous chapters we demonstrated that CIB1-KO ECs have a decrease in growth factor-induced migration, proliferation, tubule formation, monolayer resistance, and MMP2 expression. In this chapter we extend these findings further and demonstrate that CIB1-KO mouse tissue has attenuated growth factor-induced microvessel sprouting in *ex vivo* organ cultures, and in *in vivo* Matrigel plugs. Since pathological angiogenesis is highly dependent on the expression and secretion of growth factors and MMPs, we hypothesized that in addition to reduced ischemia-induced angiogenesis (Chapter 5), CIB1-KO mice may also have a decrease in tumor-induced angiogenesis. We tested this in two different xenograft tumor models, using either B16 melanoma or Lewis lung carcinoma tumor cells. Melanoma tumors that developed in CIB1-KO mice were smaller in volume, had a distinct necrotic appearance, and reduced intratumoral microvessel density. Similarly, carcinoma tumors that developed in CIB1-KO mice had decreased weight and volume, and had a different morphological appearance. Preliminary evidence also demonstrates that CIB1-KO mice, also transgenic for the PyV-mT oncogene, have a decreased number of palpable breast cancer tumor nodules and a reduced tumor burden. Thus, these findings emphasize the role of CIB1 in adult pathological angiogenesis, and suggest that it has an important role in regulating tumor growth.

6.2 Introduction

CIB1, is a 22kDa EF-hand-containing protein that was originally identified as a binding partner for the cytoplasmic tail of the platelet integrin αIIb(1). In subsequent studies, CIB1 was found to inhibit agonist-induced αIIbβ3 activation in megakaryocytes(2). But CIB1 is also widely expressed in various organs, tissues, and cell types, which suggests that it likely has additional roles independent of αIIb function(3;4). Accordingly, CIB1 also binds and regulates the activity of various proteins including the transcription factor PAX3(5), the polo-like kianses Fnk and Snk(6), the inositol 1,4,5-triphosphate receptor(7), Rac3(8), PAK1(9), and FAK(10). Among these binding partners, PAK1 and FAK have been show to regulate EC function *in vitro*, and contribute to angiogenesis *in vivo*(11-13). Therefore this led us to investigate whether CIB1 can also play a role in EC function and angiogenesis.

In previous chapters we demonstrate that CIB1 is essential for proper EC signaling and is required for various EC functions including migration, proliferation, nascent tubule formation, and monolayer resistance. Loss of CIB1 in ECs also leads to an attenuated response to angiogenic growth factors such as VEGF and bFGF, which results in a decrease in the expression of the zinc-requiring matrix-degrading proteinase MMP2. Furthermore, using different murine *in vivo* angiogenesis assays we demonstrate that although CIB1 is not essential for developmental retinal vasculogenesis and angiogenesis, CIB1-KO mice are nevertheless impaired in pathological as well as adaptive forms of ischemia-induced angiogenesis. Although this data clearly shows that CIB1 facilitates tissue recovery after

ischemic injury and participates in angiogenesis *in vivo*, the role of CIB1 in the more complex angiogenic processes is still not defined.

One such process is tumor-induced angiogenesis which is a hallmark of cancer and metastatic disease(14). This pathological form of angiogenesis was first noticed a century ago, and was hypothesized by Folkman to be the process by which tumors grow and eventually invade surrounding tissue and distant sites(15). It is now commonly accepted that neovascularization takes place in and around tumors in response to diffusible factors that tumor cells secret when they become hypoxic. This occurs as tumors exceed 1-2 mm^3 in size and O_2 is unable to diffuse efficiently to the center of the tumor(16;17). This hypoxic setting activates the heterodimeric transcription factor HIF-1 to induce expression of angiogenic growth factors and cytokines – a process that drastically alters the delicate balance between pro- and anti-angiogenic factors in the tumor microenvironment and tips the angiogenic switch in favor of angiogenesis(18;19). Hence to a large extent, pathological tumor-induced angiogenesis ensues in a growth factor-dependent manner, and this dependency could be exploited to develop effective anti-tumor therapy.

Recent successes in both pre-clinical and clinical studies have demonstrated that targeting the tumor vasculature is indeed a promising strategy for impeding tumor growth(20). One such strategy that is still under development is the selective targeting of zinc-dependent matrix degrading proteinases such as the gelatinases MMP2 and MMP9(21;22). Both MMP2 and MMP9 are secreted by most tumor cells as well as by activated tumor ECs(23). They can also hydrolyze type IV collagen that is localized in the

basement membrane and is secreted in large amounts by malignant tumor cells(24). To support this, studies have shown that MMP2 and MMP9 contribute to tumor progression and metastasis, and are essential for tumor-induced angiogenesis(25;26). Furthermore, MMP2 binds to the tumor EC-surface proteins MMP14 and integrin αvβ3 to perhaps modulate their activity and facilitate angiogenesis(27;28). Although clinical trials that have tested anti-MMP agents demonstrate that this treatment is associated with significant toxicities and untolerated side-effects, the efficacy and safety profile of selective anti-MMP2 agents has not yet been tested(22).

Here we report that in addition to participating in ischemic recovery and ischemia-induced angiogenesis (Chapter 5), CIB1 is also critical for mediating growth-factor induced angiogenesis *ex vivo* and *in vivo*. Furthermore, we observe that in different murine tumor xenograft models, tumors that develop in CIB1-KO mice are smaller and are distinctly different in gross appearance compared to tumors that develop in WT controls. Tumors in CIB1-KO mice are also more necrotic and have a decrease in intratumoral microvessel density. These observations are corroborated by our preliminary findings that suggest that CIB1-KO mice, also transgenic for the PyV-mT oncogene(29), have less tumor formation and reduced tumor burden. Thus our findings here demonstrate that CIB1 plays an important role in tumor growth and tumor-induced angiogenesis, and opens a new avenue of research in cancer biology.

6.3 Methods

Aortic ring and tibialis anterior muscle *ex vivo* cultures. Thoracic aortas were isolated from WT and CIB1-KO mice and placed in cold isolation media for at least 10 minutes. Aortas were microdissected into 1mm rings and placed between 2 layers of 250μL of GFR Matrigel (BD Biosciences) in a 24-well culture format. Polymerized Matrigel was then overlaid with 300μL growth media, or basal media supplemented with 50ng/mL bFGF or VEGF (R&D Systems). At 2, 5, and 8d, images of aortic rings and their newly formed blood vessels were collected and stitched together using PhotoFit Premium software (Tekmate, Tokyo, Japan). In digitized images, the total number of sprouting microvessels at each time point, in WT and CIB1-KO aortic rings, was counted using ImageJ as described previously (n = 8 per group). Identical procedures were used for the tibialis anterior muscle cultures, except that they were cultured only in growth media and images taken at 8d of culturing.

Matrigel plug assay. The *in vivo* Matrigel plug assay was performed as described previously with minor modifications(30;31). WT and CIB1-KO mice were subcutaneously injected with approximately 300μL of GFR Matrigel mixed with 60 U/mL of heparin (Sigma, St. Louis, MO), or with heparin plus 250ng/mL bFGF or VEGF. Fourteen days later, plugs were isolated along with adjacent skin and peritoneal muscle, fixed, and sectioned at interrupted intervals. The total number of blood-vessels infiltrating four random interrupted sections was counted in H&E stained sections using a Nikon inverted microscope, and expressed as mean values of infiltrating blood vessels per plug (n = 3 per group).

Tumor cell xenografts. Male WT and CIB1-KO mice, at least 15 weeks old, were anesthetized with 1.125% isoflurane supplemented with 2:3 oxygen-air. Rectal temperature was closely maintained at 37.0 ± 0.5˚C. Hair was removed from the hind-limb ventral adductor thigh region using depilating cream, with care to avoid erythema. Mice were given a single subcutaneous injection of either 5 x 10^5 B16 melanoma (purchased from UNC-CH Tissue Culture Facility) or 2.5 x 10^5 Lewis lung carcinoma cells (ATCC, Manassas, VA) that were impregnated in 50 uL of 50% growth factor reduced Matrigel (n = 8 - 11 mice per group).

Assessment of necrosis and tumor microvessel density. Fourteen days after tumor cell injection, tumors were isolated from animals and fixed in 2% PFA for 48h, with a solution change at 24h. Tumors were rinsed in water, placed in 70% ethanol for 48h with shaking and another change of solution at 24h. Four biopsies (~ 2-3 mm in size) were obtained from both the center and periphery of each tumor and embedded in paraffin. At least 3 interrupted sections, 50μm apart were obtained and stained with H&E or Masson's trichrome. For each tumor, one interrupted section of all four biopsies was assessed for the incidence of blood lakes and necrosis (necrosis in more than 70% of the biopsy tissue). Representative images were captured at various magnifications using a Nikon D100 camera attached to a Nikon inverted microscope. To assess capillary density, the plasma membrane of capillary ECs in tissue sections was labeled with Alexa Fluor 594 conjugated isolectin GSL-1-B4 (1:100; Invitrogen). Using a Nikon TE2000U inverted fluorescent microscope with an OrcaER, four 20X images were collected for each biopsy to yield a total of 16

images per tumor. The number of vessels with a clear lumen and < 7μm were counted by Weiping Yuan, who was blinded of animal group genotypes (n = 7 - 11 mice per group). Tumor microvessel density was reported as the average number of intratumoral microvessels.

Laser-Doppler perfusion imaging. Superficial hind-limb ventral adductor thigh regions where tumor cells were injection were monitored with noninvasive measurements using a scanning laser-Doppler perfusion imager (model LD12-IR, Moor Instruments) modified for high resolution and depth of penetration (2mm) with an 830nm-wavelenght infrared 2.5mW laser diode, 100μm beam diameter, and 15kHz bandwidth(32). Prior to measurements, anesthesia, ventilation, and temperature were controlled using the same techniques as with tumor cell injection. Blood perfusion measurements at the site of tumor injection were performed before, and at 1, 7, and 14 days after transplantation. Regions of interest (ROIs) were drawn as previously described(32), to obtain average Doppler velocity at tumor site. To control for tumor size average velocity in a ROI was normalized to the tumor Feret diameter ($\sqrt{(4*\text{Area})/\pi}$)(33;34). Data were reported in normalized mean blood perfusion units (n = 8 - 9 mice per group).

Generation of PyV-MT transgenic mice and tumor assessment. PyV-MT transgenic mice and PCR primers used to verify geneotype were a generous gift from Victoria Bautch, UNC-CH. PyV-MT male mice were crossed with CIB1-KO female mice to yield CIB1-HT/PyV-mT F1 offspring. CIB1-HT/PyV-mT male mice were then crossed a second time with either CIB1-KO or WT females to yield CIB1-KO/PyV-mT and WT/PyV-mT F2 offspring. Mice were weaned into separate cages and monitored for tumor growth.

Two to three weeks after tumor onset mice were sacrificed and the number of palpable tumor nodules was assessed.

Animal regulations. All housing, breeding, and experimental procedures performed with mice were in accordance with national guidelines and regulations, and were approved by the UNC-CH Institutional Animal Care and Use Committee (IACUC).

Statistical analysis. We compared continuous variables using the Student's t-test. For all animal studies, statistical significance was determined by an unpaired t-test for comparisons across animal groups (Wt versus CIB1-KO mice). We considered $P < 0.05$ to be significant.

6.4 Results

6.4.1 CIB1 deficiency diminishes bFGF and VEGF-induced microvessel sprouting *ex vivo*.

Expression of bFGF and VEGF is elevated several fold in ischemic tissue and in this setting they are major angiogenesis-stimulating factors(35). In Chapter 4 we showed that CIB1-KO ECs have decreased bFGF and VEGF -induced migration, proliferation, tubule formation, and MMP2 expression. In Chapter 5 we also demonstrated that CIB1-KO mice have decreased ischemia-induced angiogenesis *in vivo*. Thus to determine whether CIB1-KO

ECs have a specific defect in growth factor-induced angiogenesis we performed two types of *ex vivo* organ culture assays.

The *ex vivo* aortic ring organ culture assay is a commonly used technique that allows investigators to assess microvessel sprouting upon treatment with different types of angiogenic stimuli(36). In this case, aortic rings of WT and CIB1-KO mice were isolated and cultured for 8 days in the presence of growth media, bFGF, or VEGF. At 2 days of culturing both WT and CIB1-KO aortic rings produce microvessel sprouts. However, by 5 days of culturing a clear difference starts to emerge in the extent of microvessel sprouting from WT and CIB1-KO aortic rings. Treatment with growth media, bFGF, and VEGF all yielded fewer microvessel sprouts in CIB1-KO aortic rings (Figure 6-1A, and quantified in Figure 6-1B). This trend is even more dramatic at 8 days where CIB1-KO aortic rings cultured in growth media produced ~50% fewer microvessels, relative to WT aortic rings. Similarly, CIB1-KO aortic rings cultured in the presence of bFGF and VEGF produced ~60% fewer microvessels. At nearly all time points, WT and CIB1-KO aortic rings treated with growth media produced more microvessel sprouting than cultures that were treated with just bFGF or VEGF. This is likely because fetal bovine serum in growth media contains high concentrations of various growth factors.

We further assessed microvessel sprouting in WT and CIB1-KO mice by performing an *ex vivo* tibialis anterior muscle culture assay. In this assay microvessels produced upon angiogenic growth factors stimulation are microvascular in nature – thus more closely resembling angiogenesis *in vivo*. In this assay we observed a similar trend to that

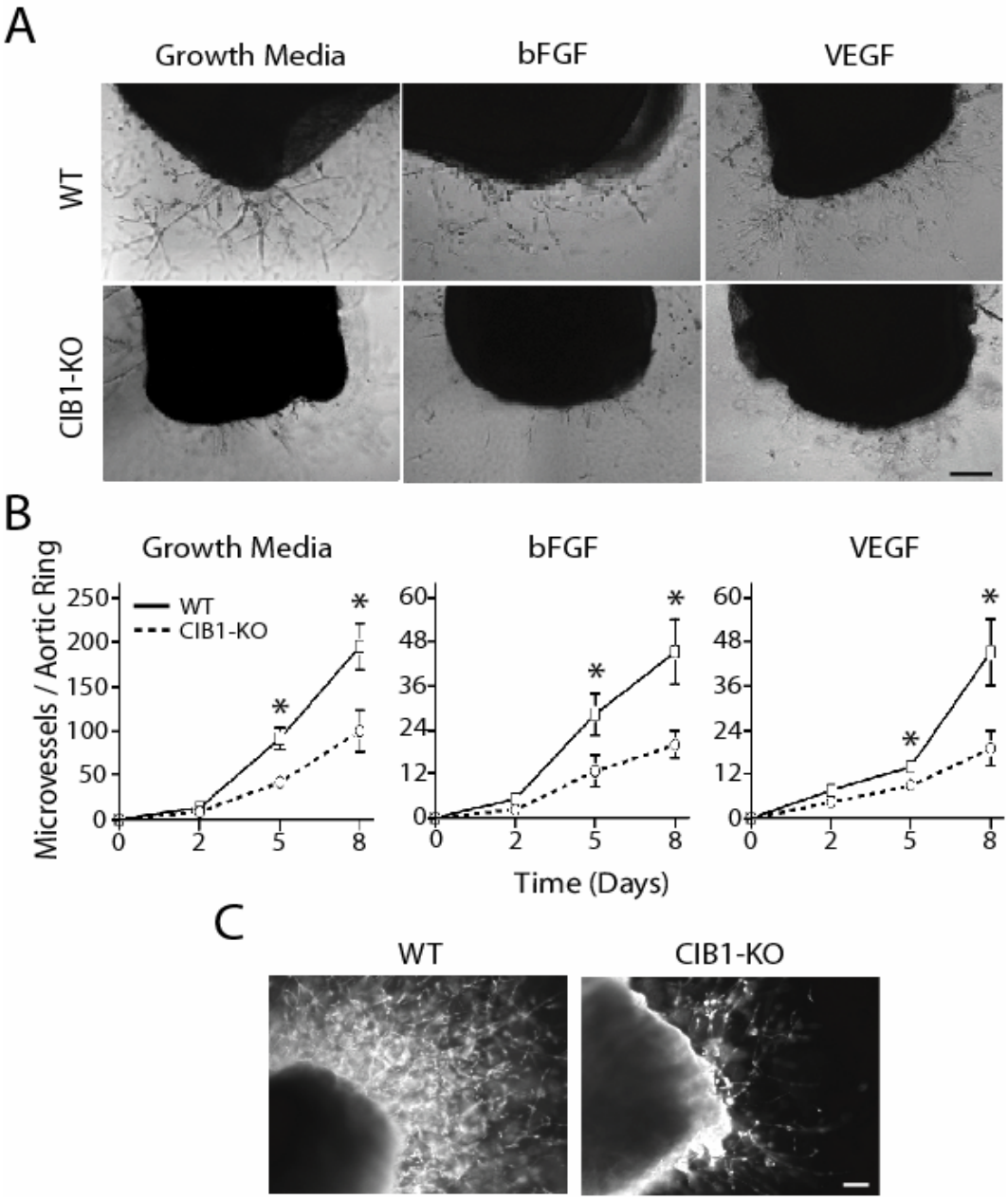


Figure 6-1. CIB1-KO aortic ring cultures demonstrate reduced growth factor-induced microvessel sprouting *ex vivo*. (**A**) Photo-stitched low-magnification DIC images of WT and CIB1-KO *ex vivo* aortic ring cultures show newly formed microvessels 8 days following treatment with growth media, bFGF, or VEGF. Scale bar, 0.2mm. (**B**) Quantification of microvessel sprouts of WT (solid line) and CIB1-KO (dotted line) aortic rings cultured in growth media, or basal media supplemented with bFGF or VEGF. Error bars are ± SEM (done in replicates of at least 4, n = 6-8 aortas per genotype). (**C**) High-magnification DIC images of microvessel sprouts in WT and CIB1-KO tibialis anterior muscle sections cultured in growth media for 8d (done in replicates of at least 2, n = 3 muscles per genotype). Scale bar, 100μm.

found using the aortic ring assay. At 8 days of culturing, CIB1-KO muscles cultured in growth media consistently form less microvessel sprouts (~50 - 60% less) compared to their WT controls (Figure 6-1C). Thus this suggests that CIB1-KO tissue have a defect in growth-factor induced angiogenesis.

6.4.2 CIB1 deficiency diminishes bFGF and VEGF-induced microvessel sprouting *in vivo*.

To determine whether CIB1-KO mice also have decreased growth-factor induced new blood vessel formation *in vivo*, we performed a subcutaneous Matrigel plug angiogenesis assay. In this assay WT and CIB1-KO mice were injected with both untreated Matrigel plugs as well as plugs that were impregnated with either bFGF or VEGF. Non-invasive Doppler flowmetery indicates that by 14 days after subcutaneous injection of plugs an increase in blood perfusion was clearly detectable (Appendix B). Accordingly, 14 days after injection of plugs, histological examination confirmed a dramatic neovascularization of the growth factor-impregnated plugs in WT mice. Although we observed cells in the growth factor-impregnated plugs subcutaneously injected in CIB1-KO mice, overall the there were ~80% less microvessels in these plugs compared to WT controls (Figure 6-2A, and quantified in Figure 6-2B).

6.4.3 Subcutaneous B16 melanoma tumors in CIB1-KO mice have increased necrosis and decreased angiogenesis.

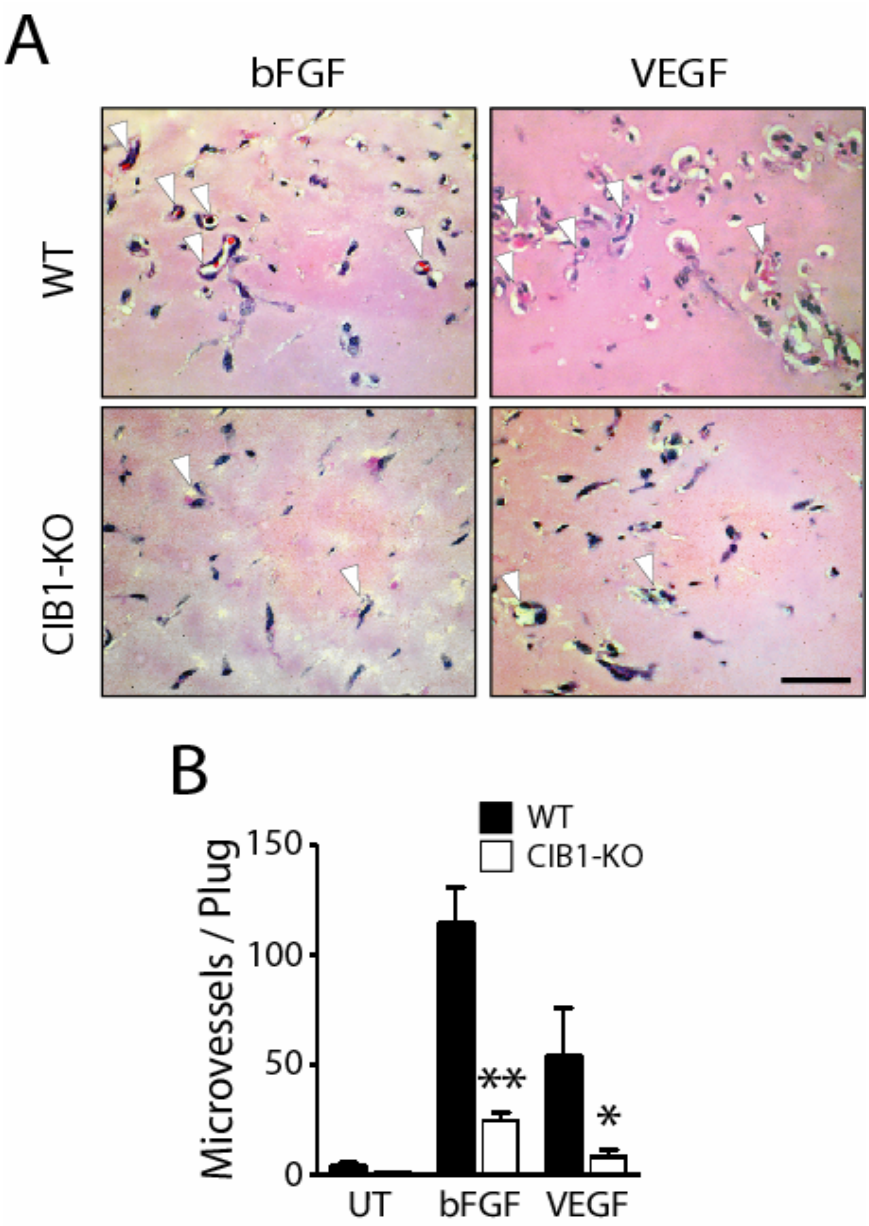
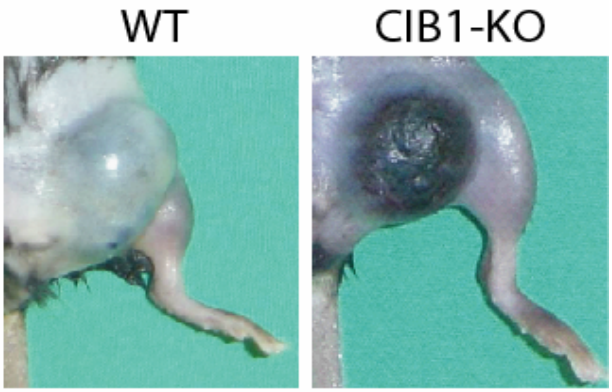


Figure 6-2. Growth factor containing Matrigel plugs have reduced microvessel sprouting in CIB1-KO mice. (**A**) Representative images of bFGF and VEGF reconstituted Matrigel plugs that were subcutaneously injected in WT and CIB1-KO mice for 14 days. Newly formed microvessels are indicated by arrowheads. Scale bar, 25μm. (**B**) Quantification of microvessels infiltrating untreated, or bFGF or VEGF reconstituted, Matrigel plugs subcutaneously injected in WT and CIB1-KO mice. Error bars represent SEM (n = 3-7 plugs per group); * $P < 0.05$, ** $P < 0.001$.

Almost all tumor cells secrete angiogenic growth factors and MMPs to stimulate the growth of new blood vessels(14;16;23). Since CIB1-KO mouse tissue demonstrates reduced growth factor-induced angiogenesis, we asked whether CIB1-KO mice also have a defect in tumor-induced angiogenesis. To examine this we xenografted WT and CIB1-KO mice with B16 melanoma tumor cells, which are known to secrete angiogenic growth factors such as VEGF, bFGF, and PDGF, but only very low levels of gelatinases MMP2 and MMP9(25;37). These rapidly dividing tumor cells were injected subcutaneously in the adductor region of the mouse hind-limb since laser Doppler flowmetery suggested that this area has relatively low blood perfusion (Appendix B). At 14 days after tumor cell injection, signs of tumor necrosis were observed in a large subset of CIB1-KO mice (Figure 6-3A). This prompted us to immediately terminate the study. The incidence of morphologically apparent melanoma tumor necrosis in CIB1-KO mice was nearly double that observed for WT mice (Figure 6-3B). Furthermore, dissection of melanoma tumors that developed in CIB1-KO reveled that they were less dense, fluid-laden, and surrounded by pockets of bleeding. Although the tumors isolated from WT and CIB1-KO mice demonstrate no significant difference in tumor weight, the melanoma tumor volume in CIB1-KO mice was reduced by 32%.

Higher incidence of tumor necrosis was observed in biopsies obtained from the center and periphery of tumors isolated from CIB1-KO mice compared to WT mice (Figure 6-4A, and quantified in Figure 6-4B). However this difference was not statistically different. Tumors isolated from CIB1-KO mice also had a slightly higher incidence of hemorrhage (observed in the form of intratumoral blood lakes) compared to WT mice,

A



B

Mouse Genotype	Necrosis	No-Necrosis	% of mice with tumor necrosis
WT (*n*=9)	3	6	33.3%
CIB1-KO (*n*=11)	7	4	63.7%

Figure 6-3. Higher incidence of morphological necrosis in B16 melanoma tumors that developed in CIB1-KO mice (A) Representative images of melanoma tumors that developed in WT and CIB1-KO mice. Study was terminated 14 days after the subcutaneous injection of tumor cells in the hind-limb adductor regions due to onset of tumor necrosis in CIB1-KO mice. **(B)** Summarizes the incidence of necrosis in 9 WT and 11 CIB1-KO mice. Sixty-seven percent of the CIB1-KO mice studied in this assay demonstrated tumor necrosis, while only 33.3% of WT mice had some signs of necrosis.

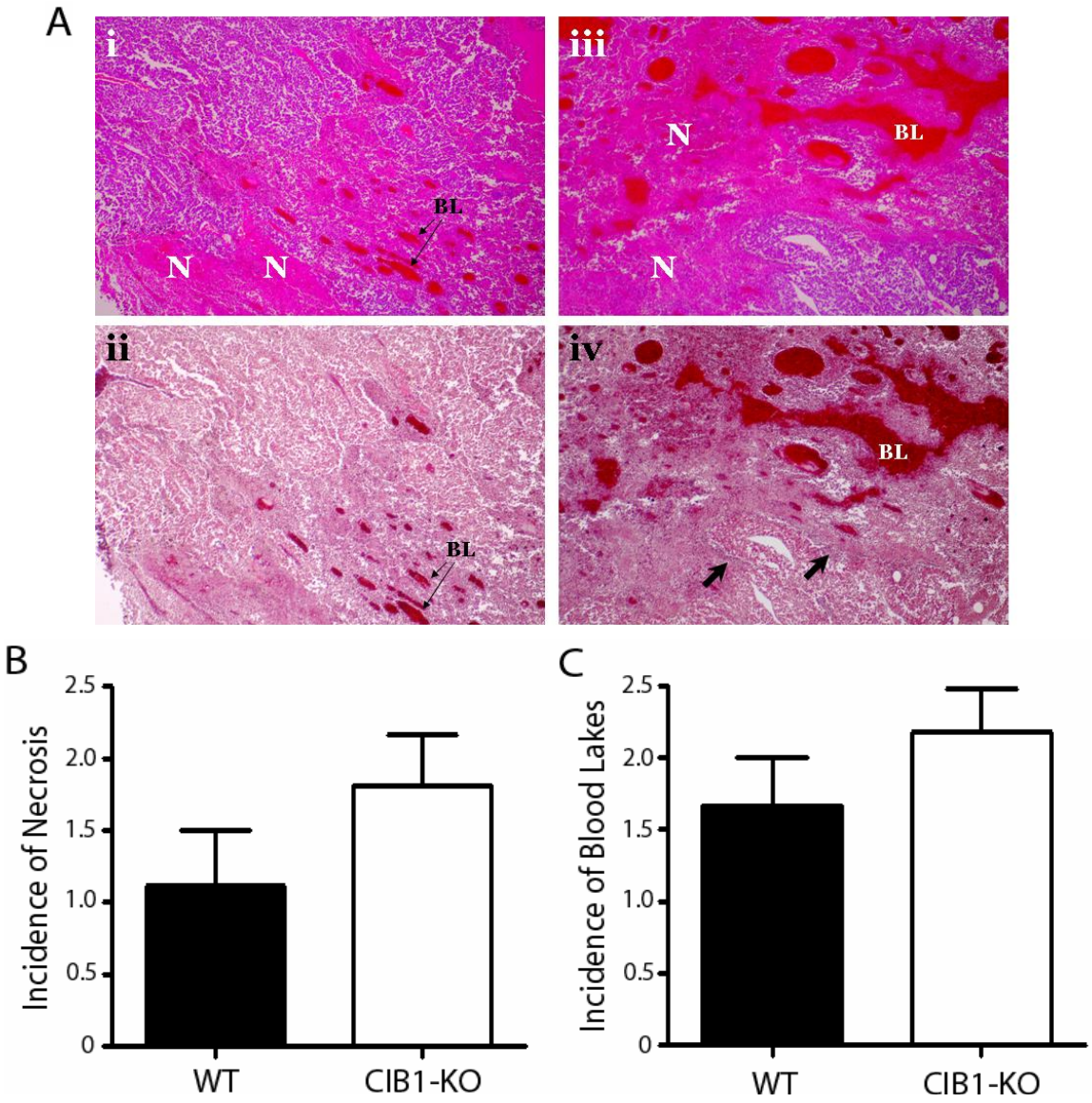


Figure 6-4. Increased necrosis and bleeding in CIB1-KO melanoma tumors. (A) Representative images of H&E (i and iii) and Masson's trichrome (MT; ii and iv) stained sections of melanoma tumors that developed in WT (i and ii) and CIB1-KO (iii and iv) mice. Necrosis (indicated by N) is observed in H&E-stained sections of tumors isolated from both WT and CIB1-KO mice, but appeared more common in tumors that developed in CIB1-KO mice. Bleeding in the form of blood lakes (BL) is also evident in H&E and MT-stained sections of tumors isolated from both groups. Blood lakes in tumors that developed in CIB1-KO mice appeared larger. MT-stained melanoma tumors isolated from CIB1-KO mice had scattered foci of fibrosis identified by arrows (iv). Scale bars, 50μm. **(B)** The incidence of necrosis in 4 biopsies per tumors per animal is elevated in CIB1-KO mice, $P = 0.06$. **(C)** A slightly increased incidence of blood lakes was also observed in tumors isolated from CIB1-KO mice, $P = 0.13$. Error bars represent SEM (n = 8-11 mice per group).

however this difference was also not statistically significant (Figure 6-4A, and quantified in Figure 6-4C). Isolectin GSL1-B4 stained microvessels were observed in tissue sections of biopsies of melanoma tumors isolated from both WT and CIB1-KO. At high magnification we observed that the microvessel density in melanoma tumors in CIB1-KO mice was 30% less than that observed in tumors isolated from WT mice (Figure 6-5A, and quantified in Figure 6-5B). Therefore, not only do xenografted melanoma tumors in CIB1-KO mice have increased necrosis, but they also have significantly reduced intratumoral neovascularization.

6.4.4 Subcutaneous Lewis lung carcinoma tumors in CIB1-KO mice have reduced tumor growth.

To expand our findings, we carried out another xenograft tumor model and subcutaneously injected WT and CIB1-KO mice with Lewis lung carcinoma tumor cells. Like B16 melanoma tumor cells, these cells secrete a variety of growth factors. However, unlike B16 melanoma tumor cells, Lewis lung carcinoma tumor cells can secrete MMP2 and MMP9 *in vivo*(25). In this assay we observed that carcinoma tumors that developed in CIB1-KO mice were decreased in weight as well as volume compared to tumors that developed in WT mice (Figure 6-6A & Figure 6-6B). Moreover, compared to carcinoma tumors that developed in WT mice, tumors that developed in CIB1-KO mice appeared paler, suggesting that there is a difference in tumor blood perfusion between the two groups (Figure 6-6C**)**. To test this we performed non-invasive Doppler flowmetery on tumors that developed in WT and CIB1-KO mice. Although this technique revealed high levels of perfusion in specific intratumoral areas, we did not observe any significant difference between WT and CIB1-KO

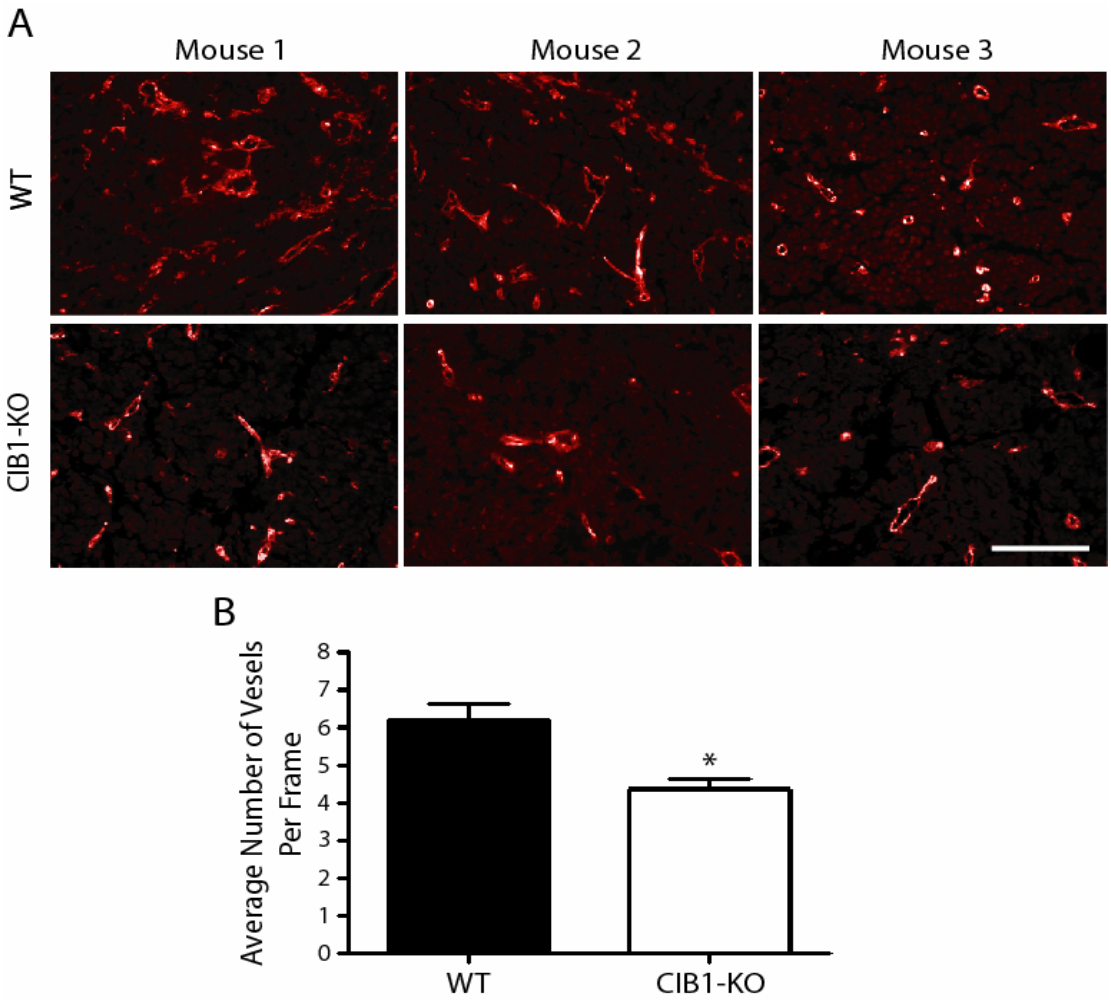


Figure 6-5. Decreased intratumoral microvessel density in melanoma tumors isolated from CIB1-KO mice. (A) Representative Isolectin GSL1-B4-stained melanoma tumor sections in three different WT and CIB1-KO mice. Scale bars, 50μm. (**B**) Quantification of microvessel density from 16 randomly collected digital frames from each tumor. Data is presented as the average number of vessels per digital frame. Error bars represent SEM (*n* = 5-9 per mouse genotype; * $P < 0.001$).

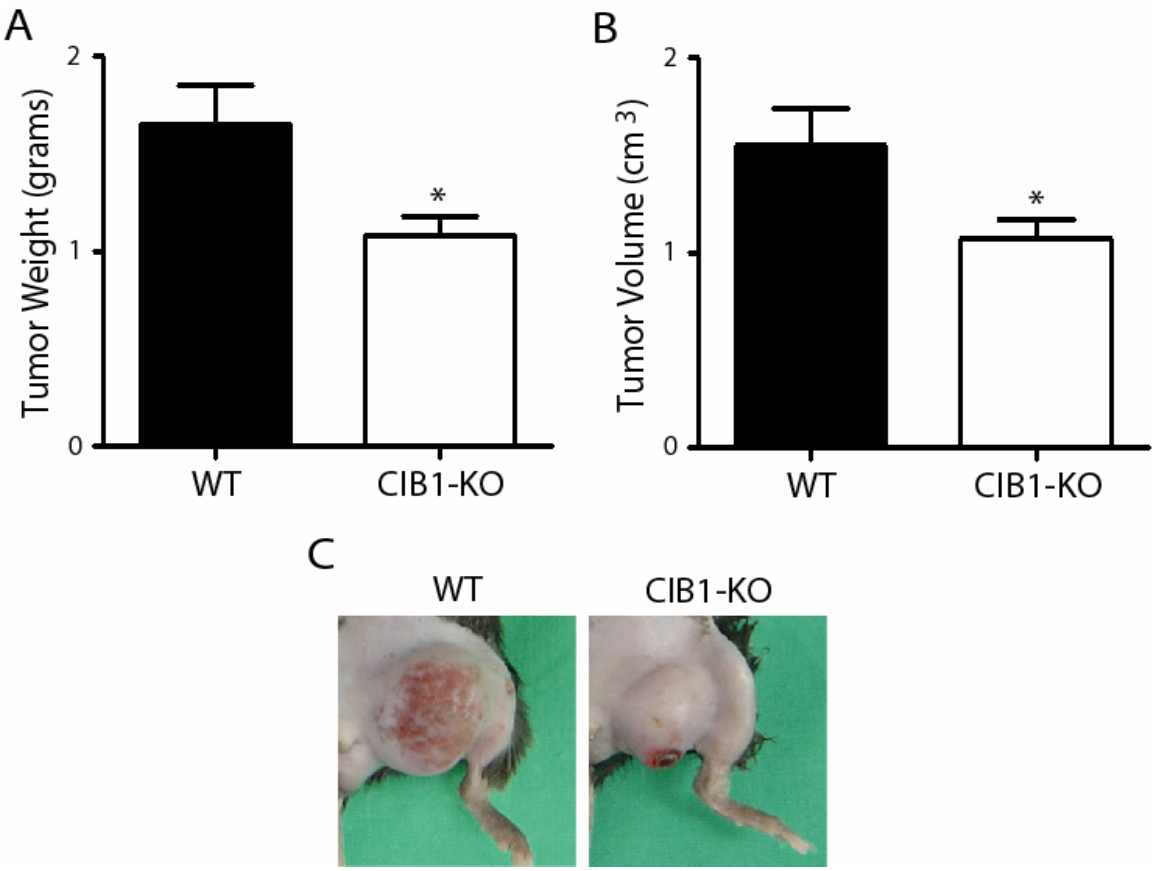


Figure 6-6. Decreased Lewis lung carcinoma tumor weight and volume in CIB1-KO mice. **(A, B)** Weight and volume of tumors that formed in CIB1-KO mice were decreased by approximately 33%. Error bars represent SEM (n = 5-9 per mouse genotype; * $P < 0.05$). **(C)** Representative images of carcinoma tumors that developed in WT and CIB1-KO mice, 14 days after subcutaneous injection of tumor cells in the adductor region of the hind-limb.

carcinoma tumor perfusion (Figure 6-7A, and quantified in Figure 6-7B). Nevertheless, we found that this technique was extremely useful in providing a real-time assessment of intratumoral blood perfusion (Appendix B).

6.4.5 CIB1-KO mice preliminarily demonstrate a reduced capacity to form PyV-mT driven breast cancer tumors.

Mice transgenic for the PyV-mT oncogene have been widely used to study mammary tumorigenesis and metastasis(38). Since our findings using subcutaneous xenograft tumor models suggest that CIB1-KO mice have reduced tumor-induced angiogenesis, increased tumor necrosis, and decreased tumor growth, we hypothesized that these features would render CIB1-KO mice more resistant to spontaneous tumor growth. To test this we crossed CIB1-KO mice with PyV-mT transgenic mice. From this cross, F2 generation WT/PyV-mT and CIB1-KO/PyV-mT transgenic mice were isolated and monitored for tumor growth. Preliminary evidence suggests that although time to tumor onset in female CIB1-KO/PyV-mT mice (~3 months) was unaffected, CIB1-KO/PyV-mT mice had smaller and less numerous breast tumors (Figure 6-8A). Approximately 14 days after tumor onset the number of palpable breast tumor nodules in CIB1-KO/PyV-mT female mice is nearly two-fold less than that observed in WT/PyV-mT mice (Figure 6-8B). These findings suggest that loss of CIB1 may inhibit aggressive breast tumor growth and decrease overall tumor burden.

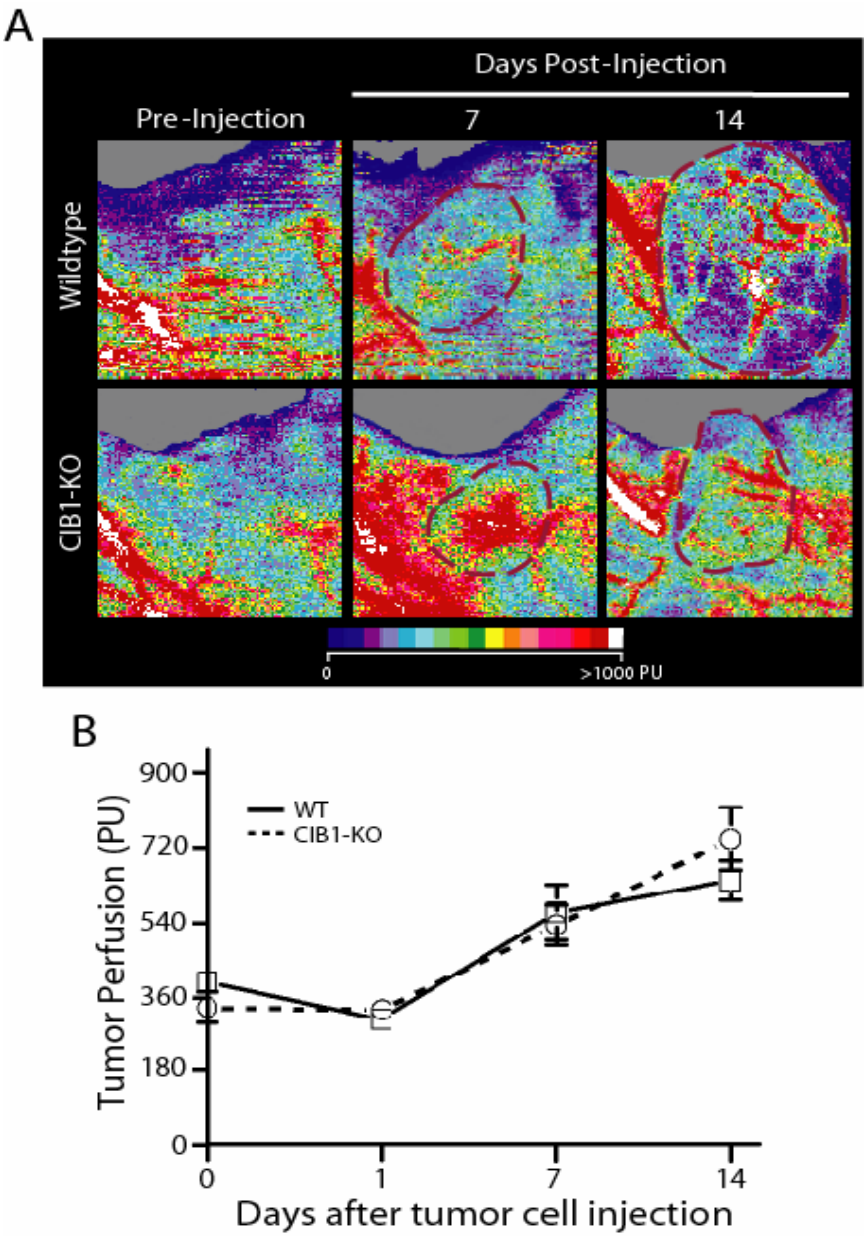


Figure 6-7. Lewis lung carcinomas growing in WT and CIB1-KO mice both demonstrated an increase in tumor blood perfusion. (**A**) Scanning laser-Doppler perfusion images of the ventral surface of upper hind-limbs where tumor cells are subcutaneously injected. Growing tumors are outlined in brown. (**B**) Quantification of perfusion was performed and corrected to Feret's diameter as described in the methods section. Data is expressed in arbitrary perfusion units (PU). Error bars are ± SEM (n = 8-9 mice per group).

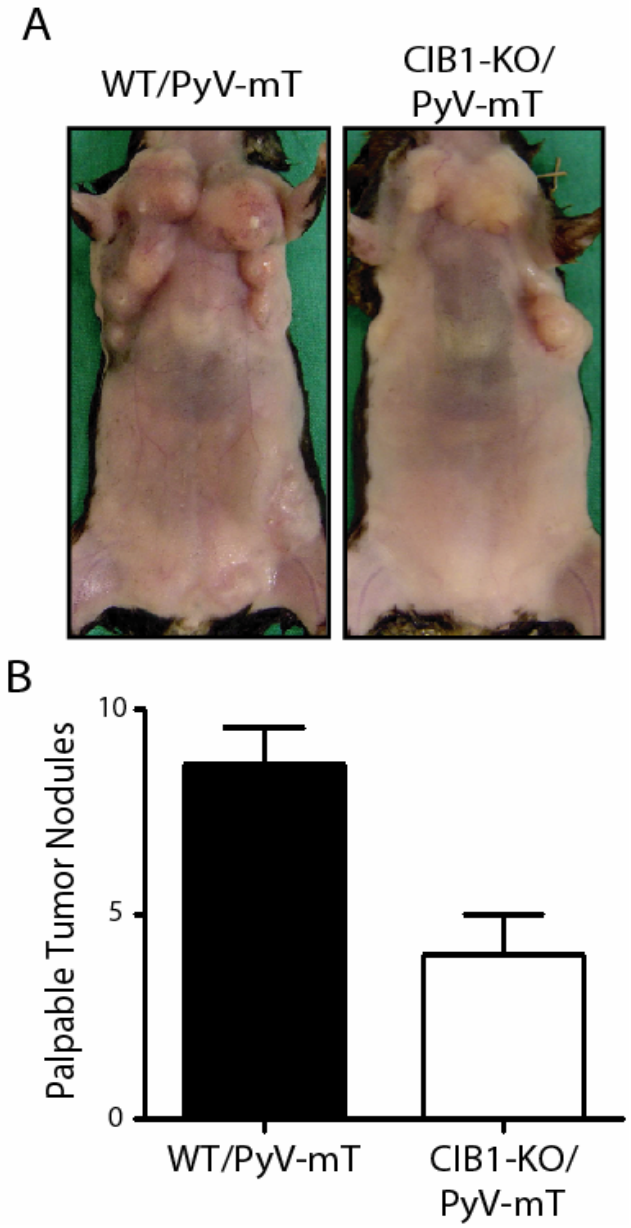


Figure 6-8. Decreased tumor formation in CIB1/PyV-mT transgenic mice. (A) Representative images of breast tumors that developed over 14 days after tumor onset in WT and CIB1-KO female littermate mice that are also transgenic for PyV-mT. CIB1-KO mice form less tumor nodules compared to WT mice. **(B)** Number of palpable breast cancer tumor nodules in CIB1/PyV-mT mice was reduced. Total breast tissue weight and volume is significantly reduced in CIB1-KO mice. Error bars are ± SEM (*n* = 2-3 mice per group).

6.5 Discussion

It is widely accepted that angiogenesis proceeds when the net balance between pro-angiogenic and anti-angiogenic molecules is tipped in favor of angiogenesis(14). Pathological ischemia- and tumor-induced angiogenesis are both facilitated by the local release of various angiogenic growth factors and MMPs(39;40). Surrounding tissues respond excessively to these stimuli and induce the aberrant formation of new blood vessels. In previous chapters we demonstrated that CIB1-KO ECs have decreased VEGF- and bFGF-induced function and signaling. We also observed a significant decrease in ischemia-induced pathological and adaptive angiogenesis in CIB1-KO mice. Therefore these findings suggest that CIB1 may have an important role in growth factor-induced angiogenesis. To test this more specifically we performed two different *ex vivo* organ culture assays and an *in vivo* Matrigel plug assay. In all these assays, CIB1-KO mouse tissue had an attenuated response to VEGF- and bFGF-induced microvessel formation, suggesting once again that CIB1 is necessary for growth factor-induced angiogenesis and angiogenesis *in vivo*.

Previous reports have demonstrated that in addition to angiogenic growth factors, MMP2 expression and secretion is essential for ischemia- and tumor-induced angiogenesis(23;40). We demonstrate (in Chapter 4) that CIB1 can regulate VEGF- and bFGF-induced MMP2 expression. Therefore, to test whether this defect can affect tumor-induced angiogenesis *in vivo*, we subcutaneously injected CIB1-KO mice with tumor cells that endogenously expression very low levels of MMP2 (B16 melanoma tumor cells) or with tumor cell that express MMP2 at high levels (Lewis lung carcinoma cells)(25;37). Our

observations clearly demonstrate that in an MMP2 deficient environment, B16 melanoma tumors that develop in CIB1-KO mice are smaller in volume, and morphologically and histologically more necrotic. In addition, melanoma tumors that developed in CIB1-KO mice demonstrate reduced microvessel density, thus providing evidence that CIB1-KO mice have reduced tumor-induced angiogenesis. On the other hand, Lewis lung carcinoma tumors that developed in CIB1-KO mice did not present with gross tumor necrosis. Perhaps endogenous expression of MMP2 by the carcinoma tumor cells is able to compensate for defects in MMP2 expression by CIB1-KO ECs, therefore enhancing tumor survival. Nevertheless, carcinoma tumors that develop in CIB1-KO mice are smaller (have decreased weight and volume), suggesting that other mechanisms in addition to EC expression of MMP2 contribute to xenograft tumor growth. In Chapter 4 we demonstrate that CIB1-KO ECs have attenuated PAK1 activation and decreased migration, proliferation, and tubule formation. It is possible that any or all of these defects also contribute to the decrease in xenograft tumor growth in CIB1-KO mice.

Another possible mechanism that may have contributed to our observations in the xenograft tumor assays is the reduced capacity for CIB1-KO ECs to efficiently form intracellular junctions. In Chapter 4 we observed decreased monolayer resistance in CIB1-KO ECs, suggesting that CIB1 was necessary for proper EC monolayer permeability. Here we observe that B16 melanoma tumors in CIB1-KO mice are more fluid-laden and are surrounded by pockets of hemorrhage and bleeding. Although it is not statistically significant, intratumoral blood lakes also occur at a slightly higher incidence in melanoma tumors that develop in CIB1-KO mice. These observations imply that tumor permeability is

increased in CIB1-KO mice. Related to this, we also observed in preliminary gene expression arrays of CIB1-KO ECs a decrease in expression of EC adhesion molecules such as PECAM1 and VE-Cadherin (Appendix A). Not only are these molecules important for maintaining EC monolayer integrity and regulating other EC functions (such as migration and tubule formation), but they can also affect tumor growth(41-43). For example, local administration of anti-murine antibodies for PECAM1 and VE-Cadherin can inhibit both growth factor- and tumor-induced angiogenesis *in vivo*(41;44). Thus, like PECAM1-KO mice (which are fertile and viable(45)) CIB1-KO mice may also have an underlying endothelial permeability defect that does not manifest overly. Depending on its severity it can decrease tumor permeability and growth, as well as tumor-induced angiogenesis. An extensive study to determine whether or not CIB1-KO mice have altered endothelial permeability is currently underway.

We also preliminarily observe a decreased in tumor growth in the breast tissue of CIB1-KO/PyV-mT transgenic mice. These observations support our findings in the B16 melanoma and Lewis lung carcinoma xenograft tumor assays. Since our previous findings also demonstrate that CIB1-KO mice have decreased pathological angiogenesis, it is possible that we observe reduced tumor burden in CIB1-KO/PyV-mT mice as a result of decreased tumor-induced angiogenesis. However, we acknowledge that there may be other factors that contribute to our observations in CIB1-KO/PyV-mT mice. For example, it is possible that CIB1 has a direct role in breast cancer tumor cells. Recently, our laboratory has identified important roles for CIB1 in epithelial, prostate, and neuroblastoma tumor cells (Tina Leisner, Bin Zhou, and Cassandra Moran, unpublished data). CIB1 was also recently found to bind

and regulate PDK1, which can activate the AGC protein kinase family, and regulate tumor cell growth, survival, and cell cycle progression. In prostate cancer cells CIB1 was found to mediate cell survival, and upon depletion of CIB1 in these cells, apoptosis was increased in response to damaging agents (Bin Zhou, unpublished data). Thus, it is possible that the lack of CIB1 may be directly affecting the rate of proliferation, invasiveness, and metastatic potential of CIB1-KO/PyV-mT breast cancer cells.

Therefore, in conclusion, we describe here a previously uncharacterized role for CIB1 in tumor growth and breast cancer. We also provide additional evidence demonstrating the crticial role of CIB1 in growth factor- and tumor-induced pathological angiogenesis. These findings, along with our observations from previous chapters, indicate that CIB1 may serve as a novel selective drug target for inhibiton of pathological angiogenesis *in vivo*.

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CHAPTER 7

Conclusion and Future Directions

7.1 Conclusion

One of the surprises of modern science arose from the human genome project. Compared to an estimated 14,000 genes in the Drosophila, the human genome has only a mere 30,000 genes(1). This indicates that the complexity of human physiology is surely not in scale with the number of genes that regulate it. This discordance is in part attributed to the complex translational machinery in humans which facilitates more alternative splicing and post-translational modifications. However, more importantly the complexity in all mammalian tissue is also likely due to a high level of molecular interplay between cellular proteins. These interactions give rise to sophisticated regulatory networks that are interconnected and affect cells and tissues in multiple ways.

This thesis represents substantial efforts to try to understand the biological function of the regulatory protein CIB1. Previously, CIB1 had only been studied in platelets, megakaryocytic, and transformed cell lines. The work presented in this thesis shows that CIB1 also plays a novel and important role in vascular ECs. *In vitro*, *ex vivo*, and *in vivo* complementary analysis, described herein, demonstrate the critical roles of CIB1 in EC function and angiogenesis. Furthermore, we show that CIB1 can affect the activation of signaling molecules such as PAK1, which regulate the angiogenic response of ECs. We also demonstrate that although CIB1 is not essential for developmental vasculogenesis and angiogenesis, it plays an important role in pathological and adaptive forms of ischemia-induced angiogenesis, as well as pathological tumor-induced angiogenesis. Thus, these findings give rise to various new insights about the biological functions of CIB1, its role in

neovasculature, as well as the process of angiogenesis in general. A summary of these considerations follows below.

7.2 CIB1 is ubiquitously expressed but is not essential for tissue growth and development.

CIB1 is expressed to a high degree in platelets(2), and megakaryocytes(3), as well as various transformed cell lines such as REF52 fibroblasts(4), HeLa ovarian epithelial cancer cells (Tina Lesiner, unpublished data), SK-N-SK neuroblastoma cells (Cassandra Moran, unpublished data), PC3 prostate cancer cells (Bin Zhao, unpublished data), and cells of megakaryocytic/erythrocytic linage (DAMI, Meg 01, K562, and HEL)(5). CIB1 mRNA and protein is also detected in nearly all human and mouse organs, and between the two species it is found in the testis, heart, brain, lung, liver, skeletal muscle, kidney, pancreas, and bone marrow(5;6). We expand this list further and demonstrate here that CIB1 mRNA is also expressed in the mouse embryo and is present in primitive mouse embryonic structures such as the yolk sac and placenta. For the first time, we also demonstrate that CIB1 is expressed in various species and types of vascular ECs. Thus collectively these findings clearly demonstrate that CIB1 is ubiquitously expressed in human and mouse tissue, and implies that CIB1 may have greater roles than previously thought.

Mice that are deficient in CIB1 are viable and reach adulthood(7). In fact, besides a male sterility defect, CIB1-KO mice appear to be phenotypically normal. This argues that CIB1 is not essential for normal growth and development, which is intriguing but certainly is

not a unique finding. Like CIB1-KO mice, many knockout mouse models for important regulatory proteins do not induce a lethal phenotype, and at times can yield mice that are completely normal (Chapter 2, Table 2-3). There are several reasonable explanations for why this can happen. As mentioned earlier, intracellular molecular signals in mammalian tissue are highly complex, and have numerous interconnections that work in concert with one another. Thus, redundancies in molecular signaling pathways may easily mask defects in pathways that are in parallel. This was shown to be the case for mice deficient in either MMP2 and/or MT1-MMP. MMP2 and MT1-MMP work in concert to activate other MMPs to facilitate angiogenesis, blood vessel remodeling, as well as organ growth(8). When either gene is deleted the mouse survives with only mild defects(9;10). However, when both genes are deleted a lethal phenotype results immediately following birth suggesting that MMP2 and MT1-MMP behave similarly and that at least one is required for survival(11). It is possible that CIB1 is not essential for survival, development, and growth, because other molecules that behave like CIB1 are able to mask its deficiency.

So far, various CIB1 homologs have been identified. These include the CIB1 family members CIB2 (59% similarity), CIB3 (62% similarity), and CIB4 (64% similarity)(12). Other CIB1 homologs also include calcineurin B (58% similarity) and calmodulin (56%)(2), as well as the primarily neuronally expressed KChIP1 (46% similarity), neurocalcin (46% similarity), and frequenin (43% similarity)(12). It is highly likely therefore that at least one of these proteins can compensate for the loss of CIB1 in CIB1-KO mouse tissue. Studies are currently underway to determine if the expression of any of these homologs is altered in

CIB1-KO mouse tissue. Preliminary results indicate that at least CIB3 and CIB4 mRNAs are overexpressed in CIB1-KO megakaryocytes (Jan DeNofrio, unpublished data).

7.3 Depletion of CIB1 significantly decreases various EC functions.

Various investigators have demonstrated that CIB1 can regulate the activity of a number of proteins that contribute to a diverse set of physiological functions (including DNA-repair, apoptosis, transcription, integrin activation, and adhesion; reviewed in (13)). The effects of CIB1 on cellular functions is variable. In certain situations, CIB1 has been found to dramatically alter cell function (~90% inhibition of agonist-induced αIIbβ3 activation in megakaryocytes(3)), while in other situations the effects of CIB1 are more modest (~25% inhibition of $InsP_3R$ channel activation; measured in the form of channel open probability(14)). Here, for the first time, we show that CIB1 also plays a functional role in microvascular ECs. Although most of the CIB1 functions are partial (loss of CIB1 does not completely abrogate EC function), they are nevertheless significant relative to controls. Thus we conclude from our findings that CIB1 critically regulates EC functions such as migration, proliferation, tubule formation, and monolayer permeability.

7.4 CIB1 regulates PAK1 and ERK1/2 activation in ECs, but likely affects other EC signals.

In addition to altering EC function, CIB1 can also regulate PAK1 activation in ECs. This confirms a pervious report that also shows a similar association between CIB1 and

PAK1(4). We also found that ERK1/2 activation, which is downstream of PAK1, is attenuated in CIB1-KO ECs. Therefore, from these results we conclude that the observed decrease in CIB1-KO EC function maybe at least in part affected by decreased PAK1 activation. However, we also acknowledge that additional studies are needed to confirm this signaling pathway. For example, constitutively active PAK1 should be expressed CIB1-KO ECs to determine whether any signaling or functional defects are normalized.

Furthermore, in addition to PAK1 and ERK1/2, we also acknowledge that altered CIB1-KO functions may be influenced by other signaling pathways. This is probable since CIB1 binds to various types of proteins that influence different signaling pathways(13). Studies are currently underway to determine if the known interactions between CIB1 and FAK as well as integrin α chains, can contribute to the phenotypes we observe in CIB1-KO ECs and CIB1-KO mice, *in vitro* and *in vivo*, respectively. Moreover, preliminary gene expression arrays indicate that CIB1-KO ECs have altered expression of various important genes that can greatly affect EC function and angiogenesis *in vivo* (i.e. Tie1, Tie2, PECAM1, VE-Cadherin, VEGF, fibronectin; Appendix A). Confirmations of these preliminary results are currently underway, and understanding their contributions to our observations is certainly a high priority.

7.5 CIB1 may also have a role in other cell types that contribute to angiogenesis.

The basic angiogenesis model primarily focuses on ECs (Figure 2-1), and proposes that EC functions such as proliferation, migration, and nascent tubule formation play a

central role in angiogenesis *in vivo*. However, it is becoming increasingly clear that other cell types such as fibroblasts, pericytes, and smooth muscle cells are also necessary for angiogenesis to proceed(15). For example, PDGF-β-KO mice are embryonically lethal due decreased pericyte recruitment to newly formed vessels(16;17). This in turn leads to the formation of excessive aneurisms and results in hemorrhage. Similarly, mice deficient in endoglin are embryonically lethal as a result of defective smooth muscle cell differentiation which severely limits the extent of vascular remodeling(18). A few reports have already implicated CIB1 in fibroblast migration and proliferation(4;7). Therefore, it is possible that other cell types in addition to ECs are affected by the loss of CIB1 and are contributing to the angiogenesis defects we observe in CIB1-KO mice. Since these contributions are not yet fully defined, this topic is certainly still open for further investigation.

7.6 Developmental angiogenesis is fundamentally different from adult pathological angiogenesis.

Our results show that CIB1 is required for pathological as well as adaptive forms of angiogenesis. However, our studies also confirm that the developing retinal vasculature in CIB1-KO mice demonstrates no significant defects in vasculogenesis and developmental angiogenesis. Therefore, CIB1 joins a growing number of genes that are not important for developmental and physiological angiogenesis, but are necessary for pathological forms of angiogenesis(19;20). We postulate that these differences occur because of the following reasons: i) Genes are differentially expressed between the developing embryonic vasculature and the adult endothelium, ii) Physiological angiogenesis is far more regulated than

pathological angiogenesis, and depends on the spatial and temporal expression of growth factors and angiogenesis mediators, and iii) Physiological angiogenesis is much more critical for survival, and therefore may have built in redundancies to overcome any defects that can occur during this process. Therefore, it is apparent that there are important molecular and functional differences between physiological and pathological angiogenesis. In the future, it may become increasingly popular to exploit these differences in order to enhance drug selectivity and decrease toxicities of anti-angiogenic therapies.

7.7 Angiogenesis is a graded phenomenon

Our data also adds to a growing body of evidence that shows that angiogenesis occurs in a graded fashion(21). In CIB1-KO mice we observed that pathological angiogenesis is significantly reduced. However, CIB1-KO mice always demonstrate a partial angiogenic response in each *in vivo* assay we performed. The same was also observed in knockout mouse models for a large set of genes, which include bFGF(22), MMP2(10), integrin α2(23), ICAM1(24), PKG(25), etc (see Table 2-3). Thus, this suggests that instead of an 'angiogenic switch', which implies either an 'on' or 'off' state, pathological angiogenesis could be better described as an 'angiogenic seesaw,' which more appropriately implies a graded angiogenic effect that depends on the extent of the imbalance between pro- and anti-angiogenic factors.

7.8 CIB1 is a candidate for pro-angiogenic treatment as well as anti-angiogenic treatment.

Finally our studies propose CIB1 as a novel target for pro- and anti-angiogenic therapy. In conditions such as ischemic heart disease and peripheral vascular disease, overexpression of CIB1 may facilitate an increase in angiogenesis, thus alleviating ischemia-associated tissue damage, morbidity, and mortality. On the other hand, in growing tumors, and in conditions such as diabetic retinopathy and age-related macular degeneration, inhibition of CIB1 may decrease neovascularization, thus reducing the severity and progression of the disease. One hypothetical advantage of CIB1 therapy may be that it will have little effect on physiological angiogenesis, and will instead be more selective to pathological or adaptive forms of angiogenesis. On the other hand, the challenge of delivering such therapy will to a large extent depend on our ability to selectively target the endothelium. This is because CIB1 is expressed in various cell types and tissues. Thus non-specific CIB1-targeting therapy may yield unwanted side effects. Strategies for selective targeting of the endothelium are slowly emerging and over the coming years may yield new and exciting opportunities for pro- and anti-angiogenic therapy.

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APPENDIX A

Preliminary Gene Expression Analysis of CIB1-KO ECs

Gene Name	Description	% Change
Ghr	Growth hormone receptor	-77.25
Cdh5	Cadherin 5	-59.81
Kitl	Kit ligand	-53.82
Tie1	Tyrosine kinase receptor 1	-52.33
Tek	Endothelial-specific receptor tyrosine kinase	-45.62
Itga4	Integrin alpha 4	-43.05
Pecam1	Platelet/endothelial cell adhesion molecule 1	-42.63
Fn1	Fibronectin 1	-40.40
Esam1	Endothelial cell-specific adhesion molecule	-38.87
Ccnl1	Cyclin L1	-36.04
Vegfa	Vascular endothelial growth factor A	-30.78
Hif1an	Hypoxia-inducible factor 1, alpha subunit inhibitor	-28.94
Bmp1	Bone morphogenetic protein 1	-28.59
Efnb2	Ephrin B2	-26.85
Ctgf	Connective tissue growth factor	-25.37
Igf1r	Insulin-like growth factor I receptor	-25.35
Eng	Endoglin	-22.93
Tgfb2	Transforming growth factor, beta 2	-22.48

Tgfa	Transforming growth factor alpha	-21.77
Notch1	Notch gene homolog 1 (Drosophila)	-20.30
Itga5	Integrin alpha 5 (fibronectin receptor alpha)	-17.26
Timp1	Tissue inhibitor of metalloproteinase 1	-17.13
Hif1a	Hypoxia inducible factor 1, alpha subunit	-15.87
Angpt1	Angiopoietin 1	-15.78
Mmp9	Matrix Metallopeptidase 9	-15.57
Bmpr2	bone morphogenic protein receptor, type II (serine/threonine kinase)	45.50
Mmp2	Matrix metallopeptidase 2	45.88
Notch2	Notch gene homolog 2 (Drosophila)	46.61
Tgfbr2	Transforming growth factor, beta receptor	46.66
Tgfb3	Transforming growth factor, beta 3	47.56
Thbs2	Thrombospondin 2	47.57
Vcam1	Vascular cell adhesion molecule 1	50.05
Pdgfb	Platelet derived growth factor, B polypeptide	54.02
Plat	Plasminogen activator, tissue	63.73
Itgb3bp	Integrin beta 3 binding protein (beta3-endonexin)	65.81
Vegfc	Vascular endothelial growth factor C	80.88
Thbs3	Thrombospondin 3	85.75
Flt1	FMS-like tyrosine kinase 1	90.31
Plat	Plasminogen activator, tissue	90.59
Angpt4	Angiopoietin 4	98.63
Bmpr1a	Bone morphogenetic protein receptor, type 1A	103.14
Pdgfra	Platelet derived growth factor receptor, alpha	112.75

Egfr	Epidermal growth factor receptor	113.07
Fgf10	Fibroblast growth factor 10	128.68
Cdh11	Cadherin 11	148.13
Tnc	Tenascin C	226.09
Ptn	Pleiotrophin	1324.54

Percent change represents the relative level of gene expression from male CIB1-KO MHECs versus male WT MHECs. Total RNA was isolated using a Qiagen RNeasy Mini Kit (Qiagen, Austin, TX) from serum starved duplicate MHEC cultures. RNA purity and quality were confirmed, and labeled by RNA amplification method at UNC-CH Genomics Core Facility. Labeled RNA was hybridized to 4x44K mouse genome microarrays (Aglient, Santa Clara, CA) and scanned with an Aglient scanner. Normalized single channel ratios were obtained and averaged for each gene. Percent change in gene expression in CIB1-KO MHECs was calculated, and a subset of the most dramatically changed genes is presented above. A negative value indicates lower gene expression and a positive value indicates higher gene expression. Also provided are the common gene name, and the description of the gene product.